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(71) Applicant (*for all designated States except US*): GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): TAI WAI FEI, David [GB/US]; 2409 Palmer Avenue, Belmont, CA 94002 (US). LOWE, John [US/US]; 396 Michelle Lane, Daly City, CA 94015 (US). JARDIEU, Paula [US/US]; 854 Hilldale Avenue, Berkeley, CA 94708 (US).

(74) Agents: LOVE, Richard, B. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).

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(54) Title: METHODS FOR DIAGNOSIS OF ALLERGY AND SCREENING OF ANTI-ALLERGY THERAPEUTICS

(57) Abstract

Methods for diagnosis of allergic disease in a patient are provided. Also provided are methods of screening anti-allergy therapeutics and IgE antagonists.

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METHODS FOR DIAGNOSIS OF ALLERGY AND SCREENING
OF ANTI-ALLERGY THERAPEUTICS

BACKGROUND OF THE INVENTION

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FIELD OF THE INVENTION

This invention relates to the field of diagnosis of allergic disease, and to the screening of therapeutics for the treatment of allergic disease.

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DESCRIPTION OF THE BACKGROUND AND RELATED ART

Three major techniques have been used in the diagnosis of allergic disease: skin tests, assays of IgE serum levels, and histamine release tests. Skin tests have

15 represented the primary diagnostic tool in allergy since their introduction in 1865. The classical skin test in atopy is the Type I wheal and flare reaction in which antigen introduced into the skin leads to the release of preformed mediators, increased vascular permeability, local

20 edema and itching. Such skin tests can provide useful confirmatory evidence for a diagnosis of specific allergy that has been made on clinical grounds. However, when improperly performed, skin tests can lead to falsely positive or negative results. The main limitation of the

25 skin test is that a positive reaction does not necessarily mean that the disease is allergic in nature, as some non-allergic individuals have specific IgE antibodies that produce a wheal and flare reaction to the skin test without any allergic symptoms.

30   The IgE-mediated false positive phenomenon observed in skin tests is not observed in *in vitro* methods for assaying allergen-specific IgE in patient serum (see Homburger and Katzmann, "Methods in Laboratory Immunology: Principles and Interpretation of Laboratory Tests for Allergy," in <u>Allergy</u>

35 <u>Principles and Practice</u>, Middleton *et al.*, eds, Mosby, pub., 4th Edition, vol. 1, chap. 21, pp. 554-572(1993)) Typically, allergen-specific IgE levels are measured by a radioallergosorbent test (RAST) wherein a patient's serum

is incubated with antigen-coated sorbent particles,
followed by detection of the specific IgE bound to antigen
with labelled antibody (see, e.g., Schellenberg *et al.*, *J.
Immunol.*, 115: 1577-1583 (1975)).

5   Total serum IgE levels are also used in the diagnosis
of allergy. Total IgE levels can be measured by
radioimmunoassy or immunometric assay methods as described
by Homburger and Katzmann, *supra*. IgE levels are often
raised in allergic disease and grossly elevated in
10 parasitic infestations. When assessing children or adults
for the presence of atopic disease, a raised level of IgE
aids the diagnosis although a normal total IgE level does
not exclude atopy. The determination of total IgE alone
will not predict an allergic state as there are genetic and
15 environmental factors which play an important part in the
production of clinical symptoms. The value of serum IgE
level in allergy diagnosis is also limited by the wide
range of IgE serum concentrations in healthy individuals.
The frequency distribution of IgE concentrations in healthy
20 adults is markedly skewed with wide 95 percentile limits
and a disproportionate number of low IgE values.
Accordingly, in calculating the 95 percentile limits of
normal IgE levels most investigators treat their data by
logarithmic transformation, which yields upper limits for
25 normal serum IgE that are very high when compared with
arithmetic means. These high upper limits for normal serum
IgE diminish the diagnostic value of the serum IgE test in
screening for clinical allergy.
        Histamine release tests provide a means to detect
30 functional, allergen-specific IgE in patient serum.
Typically, histamine release tests imitate the allergen-
specific reaction as it occurs in the patient (see, e.g.,
under van der Zee *et al.*, *J. Allergy Clin. Immunol.*, 82:
270-281 (1988)). This response has been generated *in vitro*
35 by mixing a patient's blood with different allergens and
later measuring the amount of histamine released during
each of the subsequent allergic reactions. *In vitro*
histamine release assays originally required the isolation

of leukocytes from whole blood and/or various extractions
of free histamine. Leukocyte histamine release tests were
subsequently refined and automated to avoid cell isolation
and histamine extraction (see, e.g., Siraganian et al., J.
5   Allergy Clin. Immunol., 57: 525-540 (1976)). At present,
commercially available leukocyte histamine release testing
kits permit up to 100 separate determinations with 2.5 ml
of whole blood. However, blood samples cannot be stored
for more than 24 hours prior to assay. In addition, the
10   tests produce false positive results due to non-specific
histamine release produced by toxicity of the allergen
extracts or other factors. Also, a quality control study
has reported considerable interlaboratory variability in
the measurement of histamine (Gleich and Hull, J. Allergy
15   Clin. Immunol., 66: 295-298 (1980)).

In a minority of patients with allergic symptoms,
positive skin tests and clearly detectable IgE antibodies,
no *in vitro* histamine release can be obtained from the
patients' basophil leukocytes with allergen. This
20   phenomenon makes it impossible to interpret the results of
a histamine release test if positive controls are not
available and limits the usefulness of the test in
diagnosing allergic disease. Levy and Osler, J. Immunol.,
99: 1062-1067 (1967) reported that leukocytes from only 20
25   to 30% of non-allergic individuals exhibit histamine
release upon passive sensitization with allergen-specific
IgE followed by allergen challenge *in vitro*. Ishizaka et
al., J. Immunol., 111: 500-511 (1973) expanded the
usefulness of the test by showing that the incubation of
30   leukocytes with deuterium oxide (D_2O) enhanced the histamine
release induced by passive sensitization of leukocytes with
anti-ragweed serum and challenge with ragweed antigen.
Prahl et al., Allergy, 43: 442-448 (1988) reported the
passive sensitization of isolated, IgE-deprived leukocytes
35   from non-allergic individuals with serum from a non-
releasing allergic patient followed by allergen-induced
histamine release. However, the Prahl et al. method
requires isolation of control leukocytes from the whole

blood of a non-allergic donor followed by removal of IgE bound to the donor cells. Additionally, the Levy *et al.*, Ishizaka *et al.*, and Prahl *et al.* procedures are subject to the same histamine assay variation that limits the

5   usefulness of the other histamine-release tests described above.

Accordingly, it is an object of the invention to provide a convenient, reproducible and widely applicable *in vitro* test for the diagnosis of allergic disease.

10   It is another object to provide an *in vitro* procedure for assaying the bioactivities of therapeutics for the treatment of allergic disease.

It is a further object to provide an *in vitro* procedure for screening IgE antagonists.

15   These and other objects will be apparent to those skilled in the art.

SUMMARY OF THE INVENTION

Accordingly, the invention provides for a method for

20   an *in vitro* test for the diagnosis of allergic disease in a patient wherein an allergen-specific IgE in the patient's serum is detected by allowing the allergen-specific IgE to interact with (sensitize) the Fc$_\epsilon$RI$^+$ immune cells from a non-allergic donor both in the presence and absence of an

25   IgE antagonist.

One aspect of the invention is a method for diagnosis of allergic disease in a patient comprising comparing release of a pharmacological mediator in a releasing mixture with release of a pharmacological mediator in a

30   blocked releasing mixture, wherein the releasing mixture comprises a serum sample from the patient and a tissue sample from a naive donor, and wherein the blocked releasing mixture comprises a serum sample from the patient, a tissue sample from the naive donor, and an IgE

35   antagonist, and wherein both the releasing mixture and the blocked releasing mixture are admixed with an allergen of interest.

The invention provides another *in vitro* test for the diagnosis of allergy wherein an allergen-specific IgE in a patient's serum is detected by allowing the allergen-specific IgE to interact, both in the presence and absence

5   of an IgE antagonist, with a basophil or mast cell host genetically engineered to display surface expression of a $Fc_\epsilon RI$ α-subunit capable of effecting the host cell's release of a pharmacological mediator upon exposure to patient IgE and allergen. The allergen-specific IgE/$Fc_\epsilon RI$ interaction

10 is assayed by adding the allergen and measuring the concomitant release of histamine or other pharmacological mediator. Any IgE antagonist is suitable for use in the assay, including IgE antagonists that compete with $Fc_\epsilon RI$ receptor for binding to IgE and IgE antagonists that

15 compete with IgE for binding to $Fc_\epsilon RI$ receptor. The novel use of an IgE antagonist reduces false positive results due to non-specific release of pharmacological mediator or mediators.

    One aspect of the invention is a method for diagnosis
20 of allergic disease in a patient comprising

    (a) comparing release of a pharmacological mediator in a reaction mixture with release of the pharmacological mediator in a blocked reaction mixture, wherein

    (1) the reaction mixture and the blocked reaction
25 mixture comprise cells that are the progeny of a common parent cell selected from the group consisting of: any mast cell host genetically engineered to display surface expression of a $Fc_\epsilon RI$ α-subunit that is capable of mediating the host cell's release of the pharmacological mediator

30 upon induction by patient serum IgE and allergen, and any basophil cell host genetically engineered to display surface expression of a $Fc_\epsilon RI$ α-subunit that is capable of mediating the host cell's release of the pharmacological mediator upon induction by patient serum IgE and allergen,

35   (2) the reaction mixture and the blocked reaction mixture each further comprises a portion of a single serum sample from the patient, the presence or absence in the

serum sample of IgE specific for an allergen of interest
being theretofore unknown,

(3) the blocked reaction mixture further comprises an
IgE antagonist, and

5   (4) both the reaction mixture and the blocked reaction
mixture are admixed with the allergen; and

(b) determining, based on the comparison in step (a),
the presence or absence in the serum sample of IgE specific
for the allergen.

10   The invention also provides a method for an *in vitro*
test for the bioactivity of an agent as an anti-allergy
therapeutic and for the bioactivity of an agent as an IgE
antagonist wherein
the agent is assayed for its ability to block the

15   interaction between allergen-specific IgE and $Fc_{\epsilon}RI$ receptor
by contacting the allergen-specific IgE with $Fc_{\epsilon}RI^{+}$ immune
cells in the presence or absence of the agent. The
allergen-specific IgE/$Fc_{\epsilon}RI$ interaction is assayed by adding
allergen and measuring the concomitant release of histamine

20   or other pharmacological mediator.

One aspect of the invention is a method for assaying
the bioactivity of an agent for blocking IgE-induced immune
cell sensitization comprising comparing release of a
pharmacological mediator in a releasing mixture with

25   release of a pharmacological mediator in a blocked
releasing mixture, wherein the releasing mixture comprises
a tissue sample from a naive donor and a serum sample, and
wherein the blocked releasing mixture comprises a tissue
sample from the naive donor, a serum sample, and the agent,

30   and wherein both the releasing mixture and the blocked
releasing mixture are admixed with an allergen of interest.

Another aspect of the invention is a method for
screening agents for the ability to inhibit immune cell
degranulation. In this method, immune cells from a patient

35   treated with the agent or donor immune cells preincubated
with the agent *in vitro* are first mixed with allergen-
specific IgE in the presence or absence of IgE antagonist,
and the mixtures are then challenged with the allergen.

The ability of IgE antagonist to reduce histamine release indicates the ability of the agent to inhibit immune cell degranulation.

5   BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graphic depiction of the inability of rhuMAbE25 to trigger histamine release. Blood samples from 12 naive donors were presensitized with 10% RSHP and challenged with HBSS/1% BSA, 0.1 μg/ml ragweed, 10 μg/ml

10 MAE1, or 10 μg/ml rhuMAbE25 for 30 min at 37°C. The histamine released (nM) into the supernatants of the various reaction mixtures is shown as follows: HBSS/1% BSA mixtures are identified as open squares, ragweed mixtures are identified as filled squares, MAE1 mixtures are

15 identified as open triangles, and rhuMAbE25 mixtures are identified as filled circles.

Fig. 2 is a graphic depiction of the screening of ragweed specific human plasmas. Whole blood from one naive donor was presensitized separately with 7 different human

20 plasmas (A-G) taken from donors known to be allergic to ragweed in the absence (filled column) and presence (hatched column) of 1 μg/mL rhuMAbE25 prior to challenge of 0.1 μg/mL ragweed allergen. Mixtures presensitized with HBSS/1% BSA alone are represented with open columns. Each

25 column represents the mean of duplicate determinations. Plasma B (lot 42-365054) was chosen to sensitize basophils in subsequent assays. Each error bar represents the range of duplicate determinations.

Fig. 3 is a graphic depiction of the effect that

30 presensitization with 10% RSHP has on the ragweed-induced histamine release of whole blood. Heparinized blood specimens were preincubated for 2 hr at 37°C with either PBS/0.1%BSA (open column) or 10% RSHP, lot 42-365054 (hatched column), prior to challenge with ragweed allergen

35 at 0, 1, 10 and 100 ng/mL. The induced histamine release was quantitated and is expressed in terms of its concentration (nM) in the various reaction supernatants.

Each column represents the range of duplicate
determinations (open circle).

Fig. 4 is a graphic depiction of the histamine
releasing ability of blood from different naive donors.

5   Naive donor blood was screened for histamine releasing
ability suitable for use in the bioactivity assay of
rhuMAbE25. Donors' blood was sensitized by incubation with
10% RSHP in the absence (closed column) or the presence of
10 μg/mL rhuMAbE25 (hatched column) for 2 hours at 37°C

10 prior to challenge with 0.1 μg/mL ragweed allergen.
Histamine release in response to HBSS/1% BSA challenge is
represented by open columns. The induced histamine release
is expressed as a percent of total cellular histamine.
Each column represents the mean of duplicate

15 determinations.

Fig. 5 is a graphic depiction of the kinetics of
histamine release. Naive donors' blood presensitized with
10% RSHP in the presence of 0.5 μg/mL rhuMAbE25 (open
square), 1 μg/mL rhuMAbE25 (open triangle), or no rhuMAbE25

20 (closed circle) was challenged 0.1 μg/mL ragweed allergen
and incubated over a time course. Naive donors' blood
presensitized with 10% RSHP and challenged with HBSS/1% BSA
(open circle) was used as a negative control. The induced
histamine release was quantitated and is expressed in terms

25 of its concentration (nM) in the various reaction
supernatants.   The error bar represents the range of
duplicate determinations.

Fig. 6 is a graphic depiction of the decrease in
histamine release caused by lack of Ca^{+2} and Mg^{2+} in the

30 incubation medium.   Heparinized whole blood from naive
donors was diluted with either Hanks buffer (open circle)
or Hanks buffer without Ca^{+2} and Mg^{2+} (HBSS^{--}) (filled circle)
and incubated with various concentrations of rhuMAbE25 for
2 hr prior to challenge with ragweed allergen (0.1 μg/mL)

35 for 30 minutes at 37°C. Each point represents the mean of
duplicate determinations.

Fig. 7 is a graphic depiction of the effect of
incubation medium Ca^{2-} and Mg^{2+} concentration on histamine

release. Naive donor whole blood samples diluted with HBSS⁻ containing various concentrations of $CaCl_2 \cdot 2H_2O$ (open circle), $MgCl_2 \cdot 6H_2O$ (filled circle), or $CaCl_2 \cdot 2H_2O$ plus $MgCl_2 \cdot 6H_2O$ (open square) were sensitized by 10% RSHP for 2

5 hr before challenge with ragweed allergen (0.1 μg/mL) for 30 minutes at 37°C. Release of histamine in the supernatant was quantitated and expressed as percent of total cellular histamine. Each point represents the mean of duplicate determinations.

10   Fig. 8 is a graphic depiction of the effect of rhuMAbE25 concentration on ragweed-induced histamine release. Naive donors' blood was sensitized by incubation with 10% RSHP in the presence of various rhuMAbE25 concentrations for 2 hours at 37°C prior to challenge with

15 0.1 μg/mL ragweed allergen for 30 minutes at 37°C. The induced histamine release was quantitated and is expressed in terms of its concentration (nM) in the various reaction supernatants. Each point represents the mean of duplicate determinations.

20   Fig. 9 is a graphic depiction of the effect of rhuMAbE25 on the histamine release of naive donor basophils elicited with plasma from subjects allergic to dust mite. Heparinized whole blood from one naive donor was diluted 1:7 in Hanks buffer and was separately preincubated in the

25 presence (1μg/ml) or absence of rhuMAbE25 for 2 hours with seven plasmas (A to G) taken from subjects allergic to dust mite and challenged with buffer (HBSS/1% BSA) and (0.1 μg/mL) dust mite allergen for 30 minutes at 37°C. The induced histamine release was quantitated and is expressed

30 in terms of its concentration (nM) in the various reaction supernatants. Histamine release in the absence of rhuMAbE25 is depicted with open columns and histamine release in the presence of 1μg/ml rhuMAbE25 is depicted with hatched columns. Each column represents the mean of

35 two determinations (circle).

     Fig. 10 is a graph depicting the inhibition of human IgE binding to RBL48 cells by rhuMAbE25. RBL48 cells were seeded at 40,000 cells/well and cultured overnight at 37°C

in a 5% CO$_2$ incubator prior to alcohol fixation. Fixed
cells were incubated at 37°C for one hour in one of the
following media: (1) Iscove's modified Dulbecco's medium
supplemented with 10% fetal bovine serum, 2 mM glutamine,

5   and 500 μg/ml of active geneticin (sIMDM)(Gibco BRL#11811-
031); (2) sIMDM/10% RHSP; or (3) sIMDM/10% RHSP/10 μg/ml
rhuMAbE25. Following incubation, cells were rinsed 6 times
with PBS/0.05% Tween 20, and then reacted for 30 minutes at
37°C with horse radish peroxidase (HRP)-conjugated goat

10   anti-human IgE (1/10,000 dilution) followed by 30 minutes
of incubation with o-phenylenediaminedihydrochloride (OPD)
substrate at ambient temperature. Each bar represents the
mean of duplicate determinations.

     Fig. 11 is a graph depicting the effect of temperature

15   and Ca^{2+} concentration on ragweed-induced histamine release
in RBL48 cells. RBL48 cells were seeded at 40,000
cells/well and cultured overnight at 37°C in a 5% CO$_2$
incubator. Cells were preincubated at 37°C for 2 hours with
sIMDM containing 10% (v/v) RSHP and then challenged with 0

20   μg/ml, 0.001 μg/ml, 0.01 μg/ml, 0.1 μg/ml, 1 μg/ml, or 10
μg/ml ragweed allergen in 50% D$_2$O at room temperature (open
circle), in 50% D$_2$O at 37°C (filled circle), in sIMDM at
37°C (filled square), or in 50% D$_2$O/2.5 mM EDTA at 37°C
(open square). Each point represents the mean of duplicate

25   determinations.

     Fig. 12 is a graph depicting a time course of ragweed-
induced histamine release by RBL48 cells in the presence of
50% D$_2$O. RBL48 cells were seeded at 40,000 cells/well and
cultured overnight at 37°C in a 5% CO$_2$ incubator. Cells

30   were preincubated for 2 hours with sIMDM or sIMDM/10 μg/ml
rhuMAbE25 in the presence of 10% (v/v) RSHP at 37°C. After
3 washes with sIMDM, cells were challenged with one of the
following mixtures: (1) histamine release buffer (HRB) (50%
D$_2$O, 0.8% NaCl, 1.3 mM CaCl$_2$, sIMDM)(open circle); (2) HRB

35   and 0.1 μg/ml of ragweed allergen (filled circle); (3) HRB,
0.1 μg/ml of ragweed allergen, and 0.5 μg/ml rhuMAbE25
(filled square); or (4) HRB, 0.1 μg/ml of ragweed allergen,

and 1 μg/ml rhuMAbE25 (open square). Each point represents the mean of duplicate determinations.

Fig. 13 is a graph depicting rhuMAbE25 inhibition of ragweed-induced histamine release in RBL48 cells. RBL48
5 cells were seeded at 40,000 cells/well and cultured overnight at 37°C in a 5% CO_2 incubator. Cells were preincubated for 2 hours with rhuMAbE25 at 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5 and 10 μg/ml concentrations in the presence of 10% (v/v) RSHP at 37°C. After 3 washes with
10 sIMDM, cells were challenged with HRB, 0.1 μg/ml ragweed allergen, 50% D_2O for 30 minutes at 37°C. Each point represents the mean of duplicate determinations.

Fig. 14 is a graph depicting a correlation between human basophil histamine assay (HBHA) and rat mast cell
15 histamine assay (RMCHA) results. Fifteen samples of rhuMAbE25 were each tested at two doses in both the HBHA and RMCHA for ability to inhibit ragweed-induced histamine release. The rhuMAbE25 concentrations of the samples were determined from the rhuMAbE25 standard curve of the
20 respective assay. The recovered rhuMAbE25 concentrations (after correction for the dilution factors) were plotted. Simple regression analysis with a 95% confidence interval was performed using the StatView 4.0 program.

Fig. 15 discloses four graphs depicting the RMCHA
25 screening of human plasma samples from allergic subjects against an allergen panel. RBL48 cells were seeded at 40,000 cells/well and cultured overnight at 37°C in a 5% CO_2 incubator. Cells were separately sensitized with plasma samples isolated from four different allergic donors (P1,
30 P2, P3 and P4) and then challenged with HRB (A), HRB and 0.1 μg/ml standardized mite (*D. farinae*) allergen (B), HRB and 0.1 μg/ml house dust mix allergen(C), HRB and 0.1 μg/ml ragweed antigens E-B and E-C (D), HRB and 0.1 μg/ml standardized cat pelt allergen (E), or HRB and 0.1 μg/ml
35 *Alternaria tenius* allergen (F). The allergen-induced histamine release was determined as a percent of total cellular histamine for samples sensitized in the absence

(open bar) or presence (filled bar) of 1 μg/ml rhuMAbE25.
Each bar represents the mean of duplicate determinations.

Fig. 16 is a graph depicting a comparison of histamine
release induced by ragweed allergen and ragweed

5   allergen/33% D_2O in an HBHA assay. Donor's blood was
incubated with 10% RSHP for 2 hours at 37°C and then
challenged with 0.1 μg/ml ragweed allergen in the presence
or absence of 33% D_2O for 30 minutes at 37°C. A total of 27
different naive donors were used. Each point represents

10   the mean of duplicate determinations.

Fig. 17 is a graph depicting the effect of D_2O on
ragweed-induced histamine release in RBL48 cells. RBL48
cells were seeded at 40,000 cells/well and cultured
overnight at 37°C in a 5% CO_2 incubator. Cells were

15   preincubated for 2 hours with sIMDM or sIMDM/10 μg/ml
rhuMAbE25 in the presence of 10% (v/v) RSHP at 37°C. After
3 washes with sIMDM, cells were separately challenged with
various concentrations of D_2O (0%, 30%, 50%, 70%, 100%) in
each of the following mixtures: (1) HRB (filled bar); (2)

20   HRB and 0.1 μg/ml of ragweed allergen (open bar); and (3)
HRB, 0.1 μg/ml of ragweed allergen, and 10 μg/ml rhuMAbE25
(hatched bar). Each point represents the mean of duplicate
determinations.

25   ## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS
A. DEFINITIONS

:   The term "IgE antagonist" as used herein denotes a
compound capable of disrupting or blocking the interaction
between IgE and the high affinity receptor $Fc_{\epsilon}RI$ on immune

30   cells or genetically engineered cells such that the cells
do not release a pharmacological mediator or mediators in
response to allergen stimulus. Preferably, the IgE
antagonist is also characterized by its inability to induce
immune cell or genetically engineered cell release of a

35   particular pharmacological mediator or mediators. IgE
antagonists include anti-IgE antibody of any immunoglobulin
type, such as IgG, IgA, IgM, IgD and IgE, and fragments
thereof, soluble $Fc_{\epsilon}RI$ receptor and fragments thereof, anti-

Fc$_\varepsilon$RI antibody and fragments thereof, IgE variants and
fragments thereof, IgE-binding peptides, Fc$_\varepsilon$RI receptor-
binding peptides, and nonproteinaceous small molecules
capable of binding to IgE or competing with IgE for binding
5   to Fc$_\varepsilon$RI receptor.

"Anti-IgE antibody" as used herein is defined as any
antibody capable of binding to IgE such that the bound
IgE's ability to interact with Fc$_\varepsilon$RI receptor is impaired or
eliminated.

10   "Anti-IgE antibody fragment" as used herein is defined
as any portion of an anti-IgE antibody molecule that is
capable of binding to IgE such that the bound IgE's ability
to interact with Fc$_\varepsilon$RI receptor is impaired or eliminated.

"Soluble Fc$_\varepsilon$RI receptor" as used herein is defined as
15   any molecule comprising the IgE binding site in the
extracellular domain (exodomain) of the Fc$_\varepsilon$RI α chain,
wherein the molecule is capable of binding to IgE such that
the bound IgE's ability to interact with Fc$_\varepsilon$RI receptor is
impaired or eliminated.

20   The term "IgE variant" as used herein is defined as
any IgE molecule with an alteration, such as an amino acid
substitution or substitutions and/or an amino acid deletion
or deletions, which reduces or eliminates the ability of
the IgE molecule to sensitize immune cells and wherein the
25   altered IgE molecule is capable of competing with IgE for
binding to Fc$_\varepsilon$RI receptor.

The terms "IgE variant fragment" and "fragment of an
IgE variant" as used herein are defined as any fragment of
an IgE variant that is capable of competing with IgE for
30   binding to Fc$_\varepsilon$RI receptor.

The term "IgE-binding peptide" as used herein is
defined as any peptide, including soluble Fc$_\varepsilon$RI receptor and
anti-IgE antibodies and fragments thereof, that is capable
of competing with Fc$_\varepsilon$RI receptor for binding to IgE.

35   The term "Fc$_\varepsilon$RI receptor-binding peptide" as used
herein is defined as any peptide, including IgE variants
and other antibodies and fragments thereof, capable of
competing with IgE for binding to Fc$_\varepsilon$RI receptor.

The term "allergen" as used herein refers to any antigen, such as pollen, ragweed, dust mite, and cow milk antigens, capable of generating a Type I hypersensitivity reaction in a patient upon the patient's second or
5   subsequent exposure to the antigen.

The term "pharmacological mediator" as used herein denotes any compound that is released by an $Fc_{\epsilon}RI^{+}$ immune cell and that is capable of mediating an inflammatory response, such as histamine, leukotrienes, prostaglandins
10 and platelet activating factor.

The term "$Fc_{\epsilon}RI^{+}$ immune cell" as used herein is intended to refer to a cell expressing the $Fc_{\epsilon}RI$ high affinity receptor and which is capable of releasing a pharmacological mediator or mediators upon IgE-induced
15 sensitization and exposure to an antigen of interest, such as mast cells and basophils.

The term "genetically engineered" as used herein refers to cells which have been altered from their native state by the introduction of exogenous or additional
20 endogenous DNA, along with the progeny of such altered cells. Examples of genetically engineered mast cells include a mast cell that expresses an exogenous DNA encoding a $Fc_{\epsilon}RI$ α-chain that is heterologous to the host cell, a mast cell that expresses an exogenous DNA encoding
25 a $Fc_{\epsilon}RI$ α-chain that is homologous to the host cell, and a mast cell containing an exogenous DNA that upregulates the host cell's expression of endogenous DNA encoding $Fc_{\epsilon}RI$ α-chain. Examples of genetically engineered basophils include a basophil that expresses an exogenous DNA encoding a $Fc_{\epsilon}RI$
30 α-chain that is heterologous to the host cell, a basophil that expresses an exogenous DNA encoding a $Fc_{\epsilon}RI$ α-chain that is homologous to the host cell, and a basophil containing an exogenous DNA that upregulates the host cell's expression of endogenous DNA encoding $Fc_{\epsilon}RI$ α-chain.

35 The terms "patient serum" and "serum sample from a patient" as used herein are defined as any fraction of the patient's blood which contains IgE and lacks $Fc_{\epsilon}RI^{+}$ immune cells.

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The terms "serum sample" and "serum comprising IgE specific to an allergen of interest" as used herein refer to any preparation containing IgE capable of sensitizing $Fc_{\epsilon}RI^+$ immune cells such that challenge of the sensitized

5    $Fc_{\epsilon}RI^+$ immune cells with the allergen of interest induces the release of a pharmacological mediator or mediators.

The terms "donor", "naive donor", "healthy donor", and "non-allergic donor" are used interchangeably herein and are intended to refer to an individual with no allergy to

10   an allergen of interest.

The terms "tissue sample from a naive donor" and "donor tissue" as used herein are defined as any tissue, including lung tissue, whole blood, and blood fractions, taken from the naive donor which comprises $Fc_{\epsilon}RI^+$ immune

15   cells.

The terms "blood sample from a naive donor" and "donor blood" as used herein are defined as any blood fraction, including whole blood, taken from the naive donor which comprises $Fc_{\epsilon}RI^+$ immune cells.

20   The terms "releasing mixture", "sensitized mixture", "sensitized immune cells" and "sensitized donor blood" as used herein refer to a mixture of patient serum or other serum sample and donor tissue wherein any allergen-specific IgE present in the patient serum or serum sample is

25   permitted to interact with $Fc_{\epsilon}RI^+$ immune cells present in the donor tissue.

The terms "blocked releasing mixture" and "blocked sensitization mixture" as used herein refer to a mixture of patient serum or other serum sample, donor tissue, and an

30   agent that is an IgE antagonist or is a candidate IgE antagonist or candidate anti-allergy therapeutic. In the case of an IgE antagonist, the agent is preferably present in sufficient concentration to reduce the interaction between any allergen-specific IgE present in the patient

35   serum or serum sample and $Fc_{\epsilon}RI^+$ immune cells present in the donor tissue. In the case of a candidate IgE antagonist or candidate anti-allergy therapeutic, the agent is preferably present in sufficient concentration to permit assay of the

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agent's ability to disrupt or block IgE interaction with Fc_rRI^+ immune cells.

The term "bioactivity of an agent for blocking IgE-induced immune cell sensitization" as used herein is

5   defined as the agent's ability to disrupt or block IgE interaction with Fc_rRI^+ immune cells. The definition includes bioactivities that block or disrupt IgE/immune cell interaction by competing with Fc_rRI for binding to IgE and bioactivities that block or disrupt IgE/immune cell

10 interaction by competing with IgE for binding to Fc_rRI.

The term "candidate anti-allergy therapeutic" as used herein is defined as any agent that is to be evaluated for use in the treatment of allergic disease.

The term "candidate IgE antagonist" as used herein is

15 defined as any agent that is to be evaluated for use as an IgE antagonist.

The terms "IgE interaction with Fc_rRI^+ immune cells", "IgE-induced immune cell sensitization", "Fc_rRI^+ immune cell sensitization", "immune cell sensitization", and "IgE

20 interaction with Fc_rRI receptor" as used herein are defined as the binding of allergen-specific IgE to immune cell Fc_rRI receptor such that subsequent binding of allergen to Fc_rRI receptor-bound IgE induces immune cell release of pharmacological mediator or mediators.

25 B. GENERAL METHODS

  I. Detection of Allergen-Specific IgE with Naive
    Fc_rRI^+ Cells

In one aspect, the invention provides methods for the diagnosis of allergic disease. In general, the methods

30 involve the detection of allergen-specific IgE in the serum of an allergic patient using Fc_rRI^+ immune cells. More specifically, an allergen-specific IgE in the allergic patient's serum is detected by allowing the allergen-specific IgE to interact with (sensitize) the open Fc_rRI

35 receptor on basophils and/or mast cells both in the presence and absence of an IgE antagonist. The allergen-specific IgE/Fc_rRI interaction is assayed by adding the allergen and measuring the concomitant release of histamine

or other pharmacological mediator. Since the IgE antagonist blocks IgE/Fc$_\epsilon$RI interaction, a lower histamine concentration in the reaction mixture containing IgE antagonist indicates the presence of allergen-specific IgE in the patient's serum. Although histamine serves as an example of a pharmacological mediator that can be used to indicate allergen-induced, IgE-mediated immune cell release of compounds involved in an inflammatory response in much of the following discussion, it will be appreciated that the invention encompasses the use of any such pharmacological mediator, including leukotrienes, prostaglandins and platelet activating factor, in the methods defined, discussed and claimed herein.

a. Preparation of Naive Fc$_\epsilon$RI$^+$ Immune Cells

In one embodiment, the method involves contacting a patient's serum with Fc$_\epsilon$RI$^+$ immune cells from a naive donor. Any tissue sample from a naive donor that comprises Fc$_\epsilon$RI$^+$ immune cells capable of releasing a pharmacological mediator or mediators in response to IgE sensitization and allergen challenge can be used to detect the presence of allergen-specific IgE in a patient's serum. For example, a tissue sample comprising basophils can be used. Since approximately 15% of individuals have basophils which do not release histamine *in vitro*, potential donors should be screened for basophil histamine releasability that is adequate for use in the present method. Preferably, donor basophils presensitized with allergen-specific IgE release greater than 7%, and more preferably greater than 10%, of the total cellular histamine upon allergen challenge. Donors can be screened for histamine release with any allergen and corresponding IgE according to the donor screening method for ragweed-induced histamine release described in the Examples below. The potential pool of donors with acceptable releasability is increased by incubating the donor cells in the presence of deuterium oxide (D$_2$O). As shown below in the Examples, the presence of D$_2$O in the incubation medium enhances allergen-induced histamine release. D$_2$O can be effectively used at a final

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concentration in the incubation medium of about 10% to 50%, and preferably about 20% to 40%, and most preferably about 33%.

5   In a preferred embodiment, a whole blood sample from the naive donor is used. The use of whole blood advantageously minimizes the manipulation of basophils prior to assay and avoids any disruption of cell function caused by cell isolation procedures. The whole blood sample can be anti-coagulated and diluted with a buffered
10 salt solution prior to use in the assay. Since the presence of Ca^{2+} in the incubation medium has a substantial effect on histamine release (MacGlashan and Botana, _J. Immunol._, _150_: 980-991 (1993)), it is preferred that the dilution buffer contains Ca^{2+}. Alternatively, the dilution
15 buffer can contain Mg^{2+} to increase histamine release as described in the Examples below. The dilution of a blood sample in Hank's balanced salt buffer (HBSS), containing both 1.3 mM Ca^{2+} and 0.9 mM Mg^{2+}, at a ratio of about 1:2 to 1:10, and more preferably about 1:5 to 1:7, provides a good
20 environment for histamine release.

In a preferred embodiment, isolated leukocytes from the naive donor can be used. The leukocytes can be isolated and prepared according to the method of Gillespie _et al._, _J. Clin. Invest._, _51_: 2941-2947 (1972).

25 In another embodiment, the numbers of open $Fc_{\epsilon}RI$ receptors in donor cells can be increased by removal of IgE from the basophil cell surface. This technique increases the pool of potential donors with adequate receptor numbers for the assay. Bound IgE can be removed from the basophil
30 cell surface by washing the blood and by suspending the blood cells in phosphate buffer (pH 3.6) for about 5 minutes at 4°C as described by Prahl _et al._, _supra_, or by lactic acid wash as described by Pruzansky _et al._, _J. Immunol._, _13_: 1949-1954 (1983).

35 In a further embodiment, a donor tissue sample comprising mast cells can be used. Tissue samples containing mast cells can be obtained from a naive donor's lung tissue. The lung tissue can be obtained and prepared

as a cell suspension as described in Marone et al., _J. Invest. Dermatol._, _93_: 246-252 (1989).

b. Preparation of Patient Serum

Any fraction of a patient's blood which contains
5 IgE and/or lacks $Fc_\epsilon RI^+$ immune cells is suitable for use in the diagnosis of allergy according to the present method. Since it is easily obtained, a patient serum sample is a convenient fraction for analysis.

c. Sensitization of Naive $Fc_\epsilon RI^+$ Immune Cells
10   with Patient Serum

The patient's serum is added to the naive donor tissue sample with or without IgE antagonist to form a blocked sensitization mixture or a sensitized mixture, respectively. When the donor tissue is a blood sample, the
15 desirable ratio of patient serum to donor blood in the mixture will vary according to the fraction of the donor blood and the dilution thereof to be used and the dilution of the patient serum to be tested. In the case of donor whole blood diluted 1:7 with HBSS/1% BSA mixed with
20 undiluted patient serum, a suitable ratio of donor blood to patient serum is about 2:1 to 20:1, and preferably about 4:1 to 15:1, and more preferably about 9:1.

At least two mixtures of patient serum/naive donor, $Fc_\epsilon RI^+$ immune cells are compared in parallel. In one
25 mixture, IgE antagonist is used to block the binding of patient serum IgE to naive donor $Fc_\epsilon RI^+$ immune cells. In a second mixture, the patient serum is exposed to naive donor $Fc_\epsilon RI^+$ immune cells in the absence of IgE antagonist. In a preferred embodiment, an IgE antagonist that binds to IgE
30 is used in a range of concentrations in patient serum/donor tissue mixtures to titer the allergen-specific IgE in the patient serum. The analysis of the effects of a range of IgE antagonist concentrations provides a powerful tool in assessing the severity of the patient's allergic condition.
35 Optionally, serum samples known to contain allergen-specific IgE are also tested with or without IgE antagonist according to the present method in order to provide negative and positive controls, respectively.

The desired concentration of IgE antagonist in the blocked sensitization mixture will vary according to the type of IgE antagonist used. In the case of an IgE antagonist that competes with $Fc_{\epsilon}RI$ receptor for binding to
5 IgE, the preferred concentration of the IgE antagonist depends on the relative affinities of the IgE antagonist and $Fc_{\epsilon}RI$ receptor for IgE. When total blockade of IgE/$Fc_{\epsilon}RI$ receptor interaction is desired, it is preferable to use an IgE antagonist concentration that is sufficient
10 for saturation of the IgE present in the blocked sensitization mixture. In the case of an IgE antagonist that competes with IgE for binding to $Fc_{\epsilon}RI$ receptor, the preferred concentration of the IgE antagonist depends on the relative affinities of IgE and the IgE antagonist for
15 $Fc_{\epsilon}RI$ receptor. When total blockade of IgE/$Fc_{\epsilon}RI$ receptor interaction is desired, it is preferable to use an IgE antagonist concentration that is sufficient for saturation of the $Fc_{\epsilon}RI$ receptor present in the blocked sensitization mixture.

20   When an anti-IgE monoclonal antibody is used as the IgE antagonist and when blood is used as the source of naive donor $Fc_{\epsilon}RI^{*}$ immune cells, a useful range of antibody concentrations in the patient serum/donor blood mixture is preferably about 0.001 to 10 $\mu g/ml$, and more preferably
25 about 0.005 to 5 $\mu g/ml$, and still more preferably about 0.01 to 2 $\mu g/ml$.

      IgE antagonist can be mixed with the donor tissue either prior to or at the same time that the patient serum is added to the donor tissue mixture. Alternatively, the
30 patient serum can be premixed with IgE antagonist and then mixed with donor tissue.

      The donor tissue/patient serum mixtures are incubated under conditions suitable for IgE binding to $Fc_{\epsilon}RI$ receptor. In the case of donor blood/patient serum mixtures,
35 preferred conditions consist of about 2 hours of incubation at approximately 37°C in a humidified, 5% CO_2 incubator as described in the Examples below.

d.   Allergen Challenge of Sensitized $Fc_{\epsilon}RI^{+}$ Immune Cells

Following incubation, aliquots of the sensitized mixtures and blocked sensitization mixtures are challenged
5   with the allergen of interest.   Preferably, aliquots of the sensitized mixtures and blocked sensitization mixtures of approximately 50 to 200 μl, or more preferably 100 to 150 μl, in size are used for allergen challenge.   The aliquots of sensitized mixtures and blocked sensitization mixtures
10   can be placed in the wells of microtiter plates for convenient reaction with allergen and analysis.   A sufficient amount of the allergen is added to each well and the reaction mixture can be diluted with a buffer suitable for histamine release.   The amount of allergen needed
15   depends upon the concentration of IgE-bound $Fc_{\epsilon}RI$ receptor and the allergen-specific binding affinity of the IgE. Although an excess of allergen can be used in the reaction mixture, higher concentrations of allergen may cause non-specific induction of histamine release.   Allergen
20   concentrations of about 0.01 μg/ml to 100 μg/ml, and more preferably about 0.05 μg/ml to 15 μg/ml, and still more preferably about 0.1 μg/ml to 1 μg/ml, are suitable for reacting most allergens with sensitized immune cells in the present assay.

25   The incubation conditions for the allergen reaction mixtures are chosen to allow the induction of histamine release.   In a preferred embodiment, the reaction mixture is incubated for about 20 to 30 minutes at a temperature of at least 32°C, and more preferably at least 37°C ± 1°C.

30   e.   Assay of Histamine Release

The histamine release in the allergen reaction mixtures can be stopped by sharply lowering the temperature in the reaction vessels by any convenient method such as placing the microtiter plates on ice.   For each sample,
35   total cellular histamine can be measured by disrupting the cells, separating histamine from cellular debris and assaying the separated histamine.   Cells can be disrupted by any convenient method, such as sonication, Manton-Gaulin

press or osmotic pressure and freeze thawing, and the
histamine is easily separated from cellular debris, e.g.,
by filtration or centrifugation. The concentration of
histamine can be determined by a number of methods,
5   including spectrophotofluorimetry as described in Nolte et
al., Allergy, 42: 366-373 (1987), radioimmunoassay as
described by Peyret et al., J. Immunol. Methods, 90: 39-45
(1986), and enzyme immunoassays such as a binding
competition immunoassay wherein histamine competes with
10 histamine-conjugated enzyme for binding to antibody fixed
to a solid support, followed by detection of bound enzyme
with a chromogenic substrate. As described in the Examples
below, a histamine immunoassay kit available from AMAC,
Inc., Westbrook, ME can be used to measure histamine levels
15 in the present method.

     In a further embodiment of the invention, histamine
concentration can be measured by performing the histamine
releasing reactions in the presence of glass fibers
followed by detection of the histamine bound to the glass
20 fibers. Microtiter plates can be layered with glass
microfibers as described in Nolte et al. To avoid the
trapping of blood cells in the microfibers, Pipes-AMC can
be added to the wells according to the method of Nolte et
al. Next, donor blood and patient serum with or without
25 IgE antagonist are added to the wells, followed by reaction
with allergen as in the methods described above.
Interfering substances can be separated from the histamine
bound to the microfibers by washing the plates. Histamine
is released from the microfibers by addition of a suitable
30 solvent such as the $HClO_4$/o-phthaldialdehyde mixture
described in Nolte et al., and the histamine content can be
determined by any of the methods described above.

     Lastly, the percent inhibition of histamine release
caused by the IgE antagonist is calculated. The percent
35 inhibition caused by a particular concentration of IgE
antagonist can be determined by subtracting the amount of
histamine released in the presence of IgE antagonist from
the amount of histamine released in the absence of IgE

antagonist, and dividing the difference by the amount of histamine released in the absence of IgE antagonist. The ability of the IgE antagonist to reduce histamine release indicates the presence of allergen-specific IgE in the
5   patient's serum and thus identifies allergic reaction to the particular allergen. The amount of IgE antagonist needed to reduce histamine release is an indication of the clinical intensity and severity of allergic symptoms.

II. Kit for Detection of Allergen-Specific IgE in
10
Patient Serum with Naive $Fc_{\epsilon}RI^+$ Immune Cells

The testing system of the present invention can be supplied in the form of a kit, the components of which comprise a vial containing naive donor $Fc_{\epsilon}RI^+$ immune cells, a vial containing an IgE antagonist, and one or more vials
15 containing allergen(s) of interest. The naive donor $Fc_{\epsilon}RI^+$ immune cells can be provided in the form of whole blood, a blood fraction, a washed leukocyte cell suspension, or a mast cell suspension derived from a naive donor and are conveniently stored in a refrigerated container. The kit
20 can also include a vial containing a buffered salt solution suitable for promoting the release of pharmacological mediator(s) in the sensitized releasing mixtures as described in Section Ia above.

Optionally, one or more vials containing IgE specific
25 for the allergen(s) of interest are provided as a positive control. The positive control allergen-specific IgE is derived from the same animal species as the patient to be tested. The positive control allergen-specific IgE can be supplied in known concentrations to facilitate construction
30 of a standard curve useful for titering the allergen-specific IgE in patient serum.

In one embodiment, the kit includes vials containing reagents used in the analysis of pharmacological mediator(s) released by the donor naive $Fc_{\epsilon}RI^+$ immune cells.
35   In another embodiment, the kit includes a package label or package insert providing instructions as to the use of the kit in any of the methods described in Section I above.

III.   Detection of Allergen-Specific IgE in Patient
Serum with Genetically Engineered Cells

The invention further encompasses a method for
detecting allergen-specific IgE in a patient serum sample
5   by exposing the patient serum to a mast cell or basophil
genetically engineered to display surface expression of a
Fc_rRI α-chain capable of mediating the host cell's release
of a pharmacological mediator or mediators upon
sensitization of the genetically engineered cell with·
10   allergen-specific IgE in the patient's serum and exposure
to allergen.   In this system, the Fc_rRI α-chain expressed by
the genetically engineered mast cell host or basophil host
is capable of binding to any IgE in the patient's serum.
In addition, the Fc_rRI α-chain is capable of operating in
15   the host cell's pharmacological mediator releasing effector
function.   The Fc_rRI α-chain is expressed on the host cell
surface in a functional IgE receptor complex and in
sufficient numbers such that allergen-induced cross-linking
of patient IgE-bound Fc_rRI α-chain triggers mediator
20   release.   An allergen-specific IgE in the allergic
patient's serum is detected by allowing the allergen-
specific IgE to interact with the Fc_rRI receptor α-chain on
genetically engineered mast cell or basophil hosts both in
the presence and absence of an IgE antagonist.   The
25   allergen-specific IgE/Fc_rRI α-chain interaction is assayed
by adding the allergen and measuring the concomitant
release of histamine or other pharmacological mediator.
Since the IgE antagonist blocks IgE/Fc_rRI interaction, a
lower histamine concentration in the reaction mixture
30   containing IgE antagonist indicates the presence of
allergen-specific IgE in the patient's serum.

a.   Construction of Genetically Engineered
Cells

The method of the invention can be practiced with
35   any genetically engineered mast cell or basophil host.
Typically, a mast cell or basophil host genetically
engineered to express a Fc_rRI α-chain native to the animal
species of the patient is used in the method because Fc_rRI

α-chain derived from a given animal species will react with IgE derived from the same animal species. However, the invention also encompasses the use of an interspecies system wherein the Fc$_\epsilon$RI α-chain expressed by the

5   genetically engineered host cell is derived from a different animal species than that of the patient and is nevertheless capable of reacting with allergen-specific IgE present in the patient's serum and effecting the release of pharmacological mediator or mediators upon allergen

10 induction of sensitized host cells.

It will also be appreciated that the invention is not limited to the use of a Fc$_\epsilon$RI α-chain with a particular degree of homology or heterology to the expression host cell. It is the ability of the host cell/Fc$_\epsilon$RI α-chain

15 system to effect IgE-mediated release of pharmacological mediator(s) upon allergen stimulus that is necessary for practicing the present invention.   A genetically engineered mast cell or basophil suitable for use herein can be constructed by isolating mast cells or basophils

20 from appropriate tissues in an animal, cloning the mast cells or basophils in tissue culture, isolating or synthesizing DNA encoding the Fc$_\epsilon$RI α-chain of interest, cloning the Fc$_\epsilon$RI α-chain-encoding DNA into an appropriate expression vector, transfecting the mast cell or basophil

25 cell hosts with the recombinant expression vector, and detecting and isolating mast cell or basophil transfectants that express the recombinant Fc$_\epsilon$RI α-chain on the cell surface and release pharmacological mediator or mediators in response to allergen cross-linking of patient IgE/Fc$_\epsilon$RI

30 α-chain complex.

1.   Isolation of Mast Cells and Basophils

The invention can be practiced with mast cells harvested from any animal species, such as rodent, canine, feline, bovine, porcine, equine, ovine, and primate

35 species, and the like. Candidate mast cell hosts can be obtained from a number of different types of animal tissues. Mast cells can be isolated from bone marrow, peritoneal cavity fluid, spleen, fetal liver, lymph nodes,

or lung tissue harvested from an animal'. Mast cells are
then extracted from tissue samples and cloned by any
competent method known in the art, e.g. the methods of
Nabel *et al.*, Nature, 291: 332-334 (1981) or Galli *et al.*,
5 J. Cell Biol., 95: 435 (1982).

　　　　Suitable media for the culturing of cloned mast cells
are known. Commercially available media include Dulbecco's
Modified Eagle's medium (DMEM), Iscove's modified
Dulbecco's medium media (IMDM) and others known. These
10 preparations are advantageously supplemented or used with
serum, e.g. heat inactivated fetal calf serum (FCS), and
can include other sources of energy or nutrition and/or
antibiotic or antiviral agents to prevent infection.

　　　　Preferably, the cloned mast cells are maintained in
15 the presence of a growth/stimulation factor, of which
several are known, including supernatant fluid taken from
splenocytes stimulated with Con A, from cloned Lyl$^+$2$^-$
inducer T lymphocytes as described in Nagel *et al.*, *supra*,
or from WEHI-3 cells as described in Nagas *et al.*, Science,
20 212: 333 (1981). Preferred is the mast cell growth factor
obtained from cloned Lyl$^+$2$^-$ inducer T cells.

　　　　In a preferred embodiment, the candidate mast cell
line is obtained as described in U.S. Patent No. 4,559,310
to Cantor *et al.*

25 　　　　In another preferred embodiment, an immortal cell
line, e.g. a neoplastic analog of the mast cell species of
interest, is used as the mast cell host, such as the rat
mast cell line RBL 2H3, and is grown in culture as
described above.

30 　　　　The invention can be practiced with basophils
harvested from any animal species, such as rodent, canine,
feline, bovine, porcine, equine, ovine, and primate
species, and the like. Candidate basophil cell hosts can
be obtained from the blood of the animal. Leukocytes can
35 be isolated from whole blood according to any of the
procedures used for isolating naive donor leukocytes
described in Section Ia above. Basophils can be separated
from the rest of the leukocyte population by art-known

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procedures, such as reacting the leukocyte preparation with fluorochrome-labelled anti-Fc$_\epsilon$RI antibody or fluorochrome-labelled IgE followed by detection and segregation of the fluorescing cells with a flow-activated cell sorter.

5   Alternatively, basophils can be isolated from the animal species of interest according to the method of Mul et al., *J. Immunol. Meth.*, 149: 207-214 (1992). The isolated basophils are then grown in suspension cell culture according to established methods, such as those described

10   in Ishizaka et al., *J. Immunol.*, 134: 532-540 (1985).

2. Preparation of DNA Encoding Fc$_\epsilon$RI α-chain

DNA encoding the Fc$_\epsilon$RI α-chain of interest can be prepared by a variety of methods known in the art.

15   If the DNA sequence is known, the DNA can be chemically synthesized according to the methods of Engels et al., *Agnew. Chem. Int. Ed. Engl.*, 28: 716-734 (1989), such as the triester, phosphite, phosphoramidite and H-phosphonate methods. Shimizu et al., *Proc. Natl. Acad. Sci. USA*, 85:

20   1907-1911 (1988) discloses a cDNA encoding the human Fc$_\epsilon$RI α-chain and a cDNA encoding the rat Fc$_\epsilon$RI α-chain. In addition to natural sequence DNAs, non-naturally occurring DNAs encoding the Fc$_\epsilon$RI α-chain of interest are suitable for use herein. In one embodiment, codons preferred by the

25   expression host cell are used in the design of the Fc$_\epsilon$RI α-chain-encoding DNA.

Alternatively, DNA encoding the Fc$_\epsilon$RI α-chain of interest can be cloned from any cDNA library obtained from tissue believed to contain the Fc$_\epsilon$RI α-chain mRNA, generally

30   mast cells or basophils harvested from the same animal species as the intended patient. The Fc$_\epsilon$RI α-chain gene can also be obtained from a genomic DNA library. Libraries are screened with probes designed to identify the gene of interest or the protein encoded by it. The entire cDNAs

35   for the human and rat Fc$_\epsilon$RI α-chains are described in Shimizu et al. Nucleic acid encoding the Fc$_\epsilon$RI α-chains of other animal species is readily obtained by low stringency screening of genomic DNA, mast cell cDNA or basophil cDNA

libraries of the species of interest using probes having
oligonucleotide sequences from the Fc$_r$RI α-chain gene
sequences of Fig. 2 on page 1909 of Shimizu et al. These
probes usually will contain about 100 or more bases, and

5   can consist of the entire rat or human Fc$_r$RI α-chain cDNA.
The positive clones detected in the hybridization screening
are then analyzed for homology to the human and rat Fc$_r$RI α-
chains. In general, a candidate Fc$_r$RI α-chain from a given
animal species will exhibit greater than about 30% amino

10 acid sequence homology with the rat or human Fc$_r$RI α-chain
and will have the immunoglobulin-like structure of the rat
and human Fc$_r$RI α-chains as described by Shimizu et al.

Assays are conducted to confirm that the hybridizing
full length genes encode the Fc$_r$RI α-chain of interest. The

15 candidate Fc$_r$RI α-chain-encoding DNA is simply inserted into
an expression vector and transfected into a host cell that
ordinarily does not bind to IgE of the intended patient.
Transfectants that acquire the ability to bind IgE of the
intended patient thus bear the Fc$_r$RI α-chain gene of

20 interest. For example, the candidate Fc$_r$RI α-chain-encoding
DNA can be subcloned into the pSVL vector for expression by
transfected COS-7 cells, the transfectants reacted with
trinitrophenylated red blood cells and anti-dinitrophenyl
(DNP) monoclonal IgE derived from the animal species of the

25 intended patient, followed by detection of rosette
formation as generally described in Miller et al., Science,
244: 334-336(1989).

Since Shimizu et al. successfully used a full length
rat Fc$_r$RI α-chain cDNA to detect and isolate a human Fc$_r$RI

30 α-chain cDNA clone from a human mast cell cDNA library, it
will be appreciated that either the rat or the human cDNA
can be used to screen libraries for the genomic DNA or cDNA
encoding Fc$_r$RI α-chain of other animal species with a high
likelihood of success.

35   In another embodiment, polymerase chain reaction (PCR)
methodology (U.S. Patent 4,683,195; Erlich, ed., PCR
Technology, 1989) to amplify the target DNA or RNA, e.g. as
described in section 14 of Sambrook et al., Molecular

Cloning: A Laboratory Manual, Cold Spring Harbor (1989).
This method requires the use of oligonucleotide primers
that will be expected to hybridize to the Fc$_\epsilon$RI α-chain-
encoding nucleic acid of interest, and these readily are
5   selected from the rat or human Fc$_\epsilon$RI α-chain-encoding cDNAs
disclosed by Shimizu _et al._

In yet another embodiment, the Fc$_\epsilon$RI α-chain protein is
isolated from mast cells or basophils of the animal species
of interest, the α-chain protein is partially or completely
10   amino acid sequenced, the amino acid sequence information
is used to design a pool of degenerate DNA probes, and the
probe pool is synthesized and used to screen genomic DNA or
cDNA libraries for the Fc$_\epsilon$RI α-chain gene of interest. This
general procedure is described by Shimizu _et al._ for the
15   cloning of the rat Fc$_\epsilon$RI α-chain-encoding cDNA.

3.   Generation of Genetically Engineered Cells

After the Fc$_\epsilon$RI α-chain DNA is obtained, it
is inserted into an vector that is capable of expressing
20   the Fc$_\epsilon$RI α-chain DNA in the chosen mast cell or basophil
cell host. The vector construct must contain a promoter
sequence upstream of the Fc$_\epsilon$RI α-chain coding region (5' of
the coding region on the sense strand) that is capable of
effecting initiation of transcription of the Fc$_\epsilon$RI α-chain
25   DNA by the RNA polymerase endogenous to the mast cell or
basophil cell host. Optimally, the promoter sequence
strongly promotes transcription of the Fc$_\epsilon$RI α-chain DNA.
The expression vector can also contain enhancer and/or
transcription termination sequences to facilitate
30   transcription. Preferably, the host cells are transfected
with an expression vector that contains a marker gene or
are cotransfected with an expression vector and another
vector containing a marker gene in order to facilitate
detection or selection of transfectants. In a preferred
35   embodiment, the VIS expression vectors described in
Gilfillan _et al._, _J. Immunol._, _149_: 2445-2451 (1992) are
used.

Following construction of a suitable expression
vector, the mast cell or basophil cell host is transfected
with the vector DNA by any method known in the art, such as
calcium phosphate or DEAE-dextran precipitation,

5   electroporation, viral transduction, or microparticle
bombardment, and transfectants are detected or selected by
growth in a selection medium.

The transfectant mast cells or basophils are then
tested for surface expression of the $Fc_\epsilon RI$ α-chain of ·

10 interest, e.g. using the method of Miller et al. described
above or the methods described in Example 2 below. The
candidate mast cell or basophil hosts that express the $Fc_\epsilon RI$
α-chain of interest are assayed for the ability to release
pharmacological mediator(s), such as histamine, upon

15 allergen-induced cross-linking of IgE/$Fc_\epsilon RI$ α-chain complex.
The assay can be conducted by growing the candidate
genetically engineered mast cells or basophils in medium as
described in Section III(a)(1) above, contacting the
cultured mast cells or basophils with serum containing a

20 known allergen-specific IgE derived from the animal species
of the intended patient as described in Example 2 below,
contacting the sensitized mast cells or basophils with
allergen as described in Example 2 below, and measuring ·the
histamine release in the mixture by any of the methods

25 described in Section I(e) above.

Preferably, a genetically engineered mast cell or
basophil presensitized with allergen-specific IgE of the
intended patient releases greater than 7%, and more
preferably greater than 10%, of the total cellular

30 histamine upon allergen challenge. The potential pool of
genetically engineered mast cells and genetically
engineered basophils with acceptable releasability is
increased by incubating the genetically engineered cells in
the presence of deuterium oxide (D_2O). As shown below in

35 Example 2, the presence of D_2O in the medium enhances
allergen-induced histamine release. D_2O can be effectively
used at a final concentration in the incubation medium of

about 10% to 100%, and preferably about 30% to 70%, and more preferably about 50% to 70%.

In a preferred embodiment, the rat mast cell line RBL48 described in Gilfillan *et al.* is used in the methods

5   of the invention.

b.   Preparation of Genetically Engineered Cells for Assay

The genetically engineered mast cells can be cultured for use in the methods of the invention by any

10 convenient method known to be suitable for mast cell culture, such as the tissue culture methods used for mast cell cloning described in U.S. Patent No. 4,559,310 to Cantor *et al.* After the genetically engineered cells are grown to confluence, they are trypsinized, suspended in

15 culture medium, and diluted to a cell density of about 0.2 x 10^6 to 0.6 x 10^6 cells/ml, and preferably about 0.4×10^6 cells/ml. Next, the cells are seeded in the wells of microtitre plates at about 20,000 to 60,000 cells/well, and preferably about 40,000 cells/well, and cultured for about

20 1 day.

In a preferred embodiment, culture supernatants are removed, e.g., by aspiration, and the adherent cells remaining in the wells are washed one or more times with culture medium in order to reduce the presence of any

25 histamine spontaneously released by the genetically engineered cells. Following the cell washes, the culture wells receive aliquots of fresh medium or, more preferably, a salt buffer designed to enhance release of pharmacological mediator(s) by the genetically engineered

30 cells. Since the presence of Ca^{2+} in the incubation medium has a substantial effect on histamine release (MacGlashan and Botana, *supra*), it is preferred that the buffer contains Ca^{2+}. Alternatively, the buffer can contain Mg^{2+} to increase histamine release as described in Examples 1 and 2

35 below. In one embodiment, the buffer is a dilution of HBSS providing a final Ca^{2+} concentration of about 0.13 mM to 0.65 mM, and preferably about 0.18 mM to 0.26 mM, and a

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final Mg^{2+} concentration of about 0.09 mM to 0.45 mM, and preferably about 0.13 mM to 0.18 mM.

The genetically engineered basophils can be cultured for use in the methods of the invention by any convenient
5 method known to be suitable for basophil suspension cell culture, such as the method of Ishizaka et al., *supra*. Preferably, the basophil suspension culture is diluted to a concentration of about 30,000 to 90,000 cells/ml, and preferably about 50,000 to 70,000 cells/ml. The dilution
10 of the suspension culture can be performed with fresh culture medium or, more preferably, a salt buffer designed to enhance histamine release as described above.

In a preferred embodiment, the presence of spontaneously released histamine is reduced by separating
15 the genetically engineered basophils from the culture medium, e.g. by centrifugation or filtration, and washing the cells one or more times with fresh culture medium. The washed cells are then resuspended in fresh culture medium or, more preferably, in a buffered salt solution suitable
20 for promoting the release of pharmacological mediator(s) by the cells as described above. The cell suspension is adjusted to attain the desired cell concentration as described above.

In addition, the histamine releasing ability of the
25 genetically engineered cells can be increased by including a suitable concentration of D_2O (described in Section III(a)(3) above) in the suspension buffer or culture supernatant. In a preferred embodiment, the genetically engineered mast cells are cultured as described in Example
30 2 below.

c. Preparation of Patient Serum for Assay

Any fraction of a patient's blood which contains IgE and/or lacks $Fc_{\epsilon}RI^+$ immune cells is suitable for use in the diagnosis of allergy according to the present method.
35 Since it is easily obtained, a patient serum sample is a convenient fraction for analysis.

d.   Sensitization of Genetically Engineered
      Cells

The patient's serum is added to the genetically engineered cell preparation with or without IgE antagonist
5   to form a blocked reaction mixture or a reaction mixture, respectively. IgE antagonist can be mixed with the genetically engineered cell preparation either prior to or at the same time that the patient serum is added to the mixture. Alternatively, the patient serum can be premixed
10   with IgE antagonist and then mixed with the genetically engineered cell preparation. In embodiments using an adherent cell culture, the reaction mixture and the blocked reaction mixture can be formed by admixing patient serum with or without IgE antagonist to the culture supernatants.
15   In embodiments using a suspension cell culture, the reaction mixture and the blocked reaction mixture can be formed by admixing patient serum with or without IgE antagonist to the cell suspensions.

In a preferred embodiment, heparin is present in the
20   reaction mixtures at a concentration of about 1 to 10 U/ml, and preferably about 2 to 6 U/ml, and more preferably about 3 U/ml. The heparin component in the reaction mixtures is advantageously provided by obtaining or preparing the patient serum in a heparin buffer. Alternatively, a
25   heparin component can be included in the cell culture medium or cell suspension buffer used in the reaction mixtures.

At least two mixtures of patient serum/genetically engineered cells are compared in parallel. In one mixture,
30   IgE antagonist is used to block the binding of patient serum IgE to genetically engineered cells. In a second mixture, the patient serum is exposed to genetically engineered cells in the absence of IgE antagonist. In one embodiment, an IgE antagonist that binds to IgE is used in
35   a range of concentrations in patient serum/genetically engineered cell mixtures to titer the allergen-specific IgE in the patient serum. The analysis of the effects of a range of IgE antagonist concentrations provides a powerful

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tool in assessing the severity of the patient's allergic
condition. Optionally, serum samples known to contain
allergen-specific IgE are also tested with or without IgE
antagonist according to the present method in order to
5   provide negative and positive controls, respectively.

The concentrations of patient serum and IgE antagonist
used in the reaction mixtures will depend upon the $Fc_\epsilon RI$ α-
chain expression levels and cell concentrations of the
particular genetically engineered cell used, the affinity
10 of patient IgE for the $Fc_\epsilon RI$ α-chain expressed by the host
cell, and the affinity of the IgE antagonist for patient
IgE and/or the $Fc_\epsilon RI$ α-chain expressed by the host cell.
Preferably, the IgE antagonist is present in sufficient
concentration to reduce the interaction between genetically
15 engineered cells and any patient IgE contained in the
blocked reaction mixture. When total blockade of IgE/$Fc_\epsilon RI$
α-chain interaction is desired, it is preferable to use an
IgE antagonist concentration that is sufficient for
saturation of the IgE and/or $Fc_\epsilon RI$ α-chain present in the
20 blocked reaction mixture. When anti-IgE monoclonal
antibody is used as the IgE antagonist and a genetically
engineered cell monolayer is contacted with patient serum
at a final dilution of about 1:100 to about 1:2, and
preferably about 1:10, a useful range of anti-IgE
25 monoclonal antibody concentrations in the patient
serum/genetically engineered cell mixture is about 0.5 to
100 $\mu g/ml$, and preferably about 1 to 10 $\mu g/ml$, and more
preferably about 2 to 5 $\mu g/ml$. It will be appreciated that
the optimum parameters for genetically engineered cell
30 concentration, patient serum dilution, and IgE antagonist
concentration in the reaction mixtures can be easily
determined with routine experimentation for any embodiment
of the present method.

The reaction mixture and blocked reaction mixture are
35 incubated under conditions suitable for IgE binding to $Fc_\epsilon RI$
receptor. Preferred conditions consist of about 2 hours of
incubation at approximately 37°C in a humidified, 5% CO_2
incubator as described in Example 2 below.

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e.   Allergen Challenge of Sensitized Genetically
         Engineered Cells

Following incubation, the reaction mixture and
the blocked reaction mixture are challenged with the
5   allergen of interest. If attached cultures of genetically
engineered cells are used, the culture supernatant is
optionally removed, e.g. by aspiration, and replaced with a
salt buffer solution with or without D_2O (to maximize
histamine release upon allergen challenge) as described in
10   Section III(b) above. The allergen challenge is
advantageously conducted in a reaction volume of
approximately 50 to 200 μl, and preferably about 100 to 150
μl. Thus, in the case of an adherent cell culture of
sensitized cells, it is desirable to replace the culture
15   supernatant with approximately 50 to 200 μl, and preferably
about 100 to 150 μl, of the above described salt buffer.
In the case of genetically engineered cell suspensions, it
is desirable to use aliquots of approximately 50 to 200 μl,
and preferably about 100 to 150 μl, of sensitized cell
20   suspension to provide the reaction volumes for allergen
challenge.

Allergen concentrations of about 0.01 μg/ml to 100
μg/ml, and more preferably about 0.05 μg/ml to 15 μg/ml,
and still more preferably about 0.1 μg/ml to 1 μg/ml, are
25   suitable for reacting most allergens with sensitized
genetically engineered cells in the present assay.

The incubation conditions for the allergen reaction
mixtures are chosen to allow the induction of histamine
release. In a preferred embodiment, the reaction mixture
30   is incubated for about 20 to 30 minutes at a temperature of
at least 32°C, and more preferably at least 37°C ± 1°C.

f.   Assay of Histamine Release

The histamine release in the allergen reaction
mixtures can be stopped by sharply lowering the temperature
35   in the reaction vessels by any convenient method such as
placing the microtiter plates on ice. For measurement of
allergen-induced histamine release, reaction mixture
supernatants are separated from cells by filtration or

centrifugation, and the histamine in the supernatants can
be determined by any of the histamine detection assay
methods described in Section I(e) above. For measurement
of total cellular histamine, the genetically engineered
5 cells in the reaction mixtures are disrupted by any
convenient method, e.g. sonication, Manton-Gaulin press,
treatment with a detergent such as Triton X-100, or osmotic
pressure and freeze thawing, the histamine is separated
from cell debris by filtration or centrifugation, and the
10 histamine is quantitated as described above.

Lastly, the percent inhibition of histamine release
caused by the IgE antagonist is calculated. The allergen-
induced histamine release can be expressed as a percent of
total cellular histamine by dividing the allergen-induced
15 histamine value by the total cellular histamine value for
each of the blocked and unblocked reaction mixtures,
yielding the blocked percentage and unblocked percentage,
respectively. The percent inhibition caused by a
particular concentration of IgE antagonist can be
20 determined by subtracting the blocked percentage from the
unblocked percentage, and dividing the difference by the
unblocked percentage. Alternatively, the percent
inhibition can be calculated by subtracting the amount of
histamine released in the presence of IgE antagonist from
25 the amount of histamine released in the absence of IgE
antagonist, and dividing the difference by the amount of
histamine released in the absence of IgE antagonist. The
ability of the IgE antagonist to reduce histamine release
indicates the presence of allergen-specific IgE in the
30 patient's serum and thus identifies allergic reaction to
the particular allergen. The amount of IgE antagonist
needed to reduce histamine release is an indication of the
clinical intensity and severity of allergic symptoms.

IV.   Kit for Detection of Allergen-Specific IgE in
35   Patient Serum with Genetically Engineered Cells
The testing system of the present invention can be
supplied in the form of a kit, the components of which
comprise a vial containing a genetically engineered mast

cell or genetically engineered basophil, a vial containing
an IgE antagonist, and one or more vials containing
allergen(s) of interest. The genetically engineered mast
cell or basophil can be conveniently provided in frozen

5 form, in which case revitalization by appropriate known
thawing procedures is required before use. The kit can
also include a vial containing a medium comprising
essential nutrients, energy sources, growth factors, etc.,
capable of supporting the growth of the genetically

10 engineered mast cell or basophil in cell culture and/or a
vial containing a buffered salt solution suitable for
promoting the release of pharmacological mediator(s) in the
reaction mixtures as described in Section III(b) above.

Optionally, one or more vials containing IgE specific

15 for the allergen(s) of interest are provided as a positive
control. The positive control allergen-specific IgE is
derived from the same animal species as the patient to be
tested. The positive control allergen-specific IgE can be
supplied in known concentrations to facilitate construction

20 of a standard curve useful for titering the allergen-
specific IgE in patient serum.

In one embodiment, the kit includes vials containing
reagents used in the analysis of pharmacological
mediator(s) released by the genetically engineered cells.

25 In another embodiment, the kit includes a package
label or package insert providing instructions as to the
use of the kit in any of the methods described in Section
III above.

V.   Screening of IgE Antagonists

30 The invention also provides a method for assaying the
bioactivity of an agent for blocking IgE-induced immune
cell sensitization. The method can be used for screening
therapeutics for the treatment of allergic disease. The
method can also be used to screen agents with IgE

35 antagonist activity for use in the allergy diagnosis
methods described above or for any other purpose. In
general, the method involves the use of an agent to be
tested in place of the IgE antagonist in the methods for

diagnosing allergic disease described above. An agent is
assayed for its ability to block the interaction between
allergen-specific IgE and Fc_rRI receptor by contacting the
allergen-specific IgE with Fc_rRI^* immune cells in the
5 presence or absence of the agent. The allergen-specific
IgE/Fc_rRI interaction is assayed by adding allergen and
measuring the concomitant release of histamine or other
pharmacological mediator. A lower histamine concentration
in the mixture containing the agent indicates the agent's
10 IgE antagonist activity.

Any serum sample which contains IgE specific to an
allergen of interest and lacks Fc_rRI^* immune cells is
suitable for use in the assay of an agent's activity in
blocking IgE-induced immune cell sensitization.
15 Appropriate serum samples can be obtained from allergic
patients as described above. In this embodiment, an agent
can be assayed for its ability to block allergen-specific
IgE from a particular patient, providing a powerful tool
for assessing the therapeutic efficacy of different agents
20 in the particular patient. Commercially prepared plasma
products are also suitable for use in the invention, such
as the ragweed specific human plasmas (RSHP) used in the
Examples below.

Fractions of naive donor blood or other naive donor
25 tissue samples suitable for use in the assay of an agent's
activity in blocking IgE-induced immune cell sensitization
can be obtained and prepared as described in the methods
for diagnosing allergic disease above.

The serum sample is added to the naive donor tissue
30 sample with or without the agent to form a blocked
sensitization mixture or a sensitized mixture,
respectively. When whole blood or a blood fraction is used
as the donor tissue, the serum and donor blood can be mixed
in the proportions described in the methods for diagnosing
35 allergic disease above.

At least two mixtures of serum/naive donor Fc_rRI^*
immune cells are compared in parallel. In one mixture, the
agent is used to block the binding of serum IgE to naive

donor Fc$_\varepsilon$RI$^+$ immune cells. In a second mixture, the serum
is exposed to naive donor Fc$_\varepsilon$RI$^+$ immune cells in the absence
of the agent to provide a positive control. In a preferred
embodiment, a series of serum/donor tissue mixtures with a
5 range of agent concentrations are used to assess the
agent's efficacy in the treatment of allergic disease or to
determine the agent's activity as an IgE antagonist. When
the agent being tested is a monoclonal antibody and when
the donor tissue being used is whole blood or a blood
10 fraction, a useful range of antibody concentrations in the
serum/blood donor mixture is preferably about 0.001 to 10
μg/ml, and more preferably about 0.01 to 5 μg/ml, and still
more preferably about 0.01 to 2 μg/ml.

The agent can be mixed with the donor tissue either
15 prior to or at the same time that the serum is added to the
donor tissue mixture. Alternatively, the serum can be
premixed with the agent and then added to the donor tissue
mixture.

The donor tissue/serum mixtures are incubated under
20 the conditions described in the methods for diagnosing
allergic disease above.

Following incubation, aliquots of the sensitized
mixtures and blocked sensitization mixtures are challenged
with the allergen of interest. Suitable aliquot sizes,
25 amounts of allergen, and incubation conditions for the
allergen reaction mixtures are described in the methods for
diagnosing allergic disease above.

The histamine released in the allergen reaction
mixtures can be measured by any of the techniques described
30 in the methods for diagnosing allergic disease above.
Lastly, the percent inhibition of histamine release caused
by the agent is calculated. The percent inhibition caused
by a particular concentration of agent is determined by
subtracting the amount of histamine released in the
35 presence of the agent concentration from the amount of
histamine released in the absence of the agent, and
dividing the difference by the amount of histamine released
in the absence of the agent. The ability of the agent to

reduce histamine release is indicative'of the agent's efficacy in the treatment of allergic disease and/or the agent's activity as an IgE antagonist. The amount of the agent needed to reduce histamine release is an indication
5 of the agent's strength as an anti-allergy therapeutic and/or IgE antagonist.

In addition, the invention provides a method of screening agents for the ability to inhibit immune cell degranulation. In this method, immune cells from a patient
10 treated with the agent or donor immune cells preincubated with the agent *in vitro* are first mixed with allergen-specific IgE in the presence or absence of IgE antagonist, and the mixtures are then challenged with the allergen. The ability of IgE antagonist to reduce histamine release
15 would indicate the ability of the agent to inhibit immune cell degranulation. The method can be used to test an agent's ability to inhibit degranulation in both mast cells and basophils.

IgE antagonists suitable for use in the present
20 invention include anti-IgE antibodies and fragments thereof, soluble $Fc_{\epsilon}RI$ receptor, IgE variants and fragments thereof, $Fc_{\epsilon}RI$ receptor-binding peptides, IgE-binding peptides, and any nonproteinaceous small molecule capable of disrupting or blocking the interaction between IgE and
25 the $Fc_{\epsilon}RI$ receptor such that $Fc_{\epsilon}RI^{\cdot}$ immune cells do not release pharmacological mediator or mediators in response to allergen stimulus. A compound can be screened for activity as an IgE antagonist according to the above-described methods for assaying the bioactivity of a agent
30 in blocking IgE-induced immune cell sensitization.

The candidate antagonist is also screened for inability to induce immune cell histamine release. Such activity would impair the effectiveness of an IgE antagonist in the methods of the invention for diagnosing
35 allergic disease. Moreover, the ability of an agent to trigger histamine release would be likely to stimulate or exacerbate allergies in patients and is therefore an undesirable characteristic for an anti-allergy therapeutic.

The histamine release-inducing activity of an agent can be
tested by mixing sensitized immune cells with the agent
instead of allergen in the above-described methods for
diagnosing allergic disease. Sensitized immune cells mixed

5 with allergen or merely buffer serve as positive and
negative controls, respectively. In a preferred
embodiment, the agent is tested for induction of histamine
release as described in the Examples below. If the agent
fails to induce histamine release that is substantially

10 above the level of the negative control, the agent
is acceptable for use in the present invention.

VI   Generation of IgE Antagonists

In a preferred embodiment, anti-IgE antibody is used
as the IgE antagonist. The anti-IgE antibody can be any

15 type of immunoglobulin, such as IgG, IgA, IgM, IgD, and
IgE, including polyclonal and monoclonal forms of such
antibodies raised against IgE.

Polyclonal antibodies to IgE generally are raised in
animals by multiple subcutaneous (sc) or intraperitoneal

20 (ip) injections of IgE and an adjuvant. It can be useful
to conjugate IgE or a fragment containing the target amino
acid sequence from the Fc region of IgE to a protein that
is immunogenic in the species to be immunized, e.g., ·
keyhole limpet hemocyanin, serum albumin, bovine

25 thyroglobulin, or soybean trypsin inhibitor using a
bifunctional or derivatizing agent, for example,
maleimidobenzoyl sulfosuccinimide ester (conjugation
through cysteine residues), N-hydroxysuccinimide (through
lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$,

30 or $R^1N = C = NR$, where R and R^1 are different alkyl groups.

Animals ordinarily are immunized against the cells or
immunogenic conjugates or derivatives by combining 1 mg or
1 μg of IgE with Freund's complete adjuvant and injecting
the solution intradermally at multiple sites. One month

35 later the animals are boosted with 1/5 to 1/10 the original
amount of conjugate in Freund's incomplete adjuvant by
subcutaneous injection at multiple sites. 7 to 14 days
later animals are bled and the serum is assayed for anti-

IgE titer. Animals are boosted until the titer plateaus.
Preferably, the animal is boosted with the conjugate of the
same IgE, but conjugated to a different protein and/or
through a different cross-linking agent. Conjugates also
5 can be made in recombinant cell culture as protein fusions.
Also, aggregating agents such as alum are used to enhance
the immune response.

        Monoclonal antibodies are prepared by recovering
spleen cells from immunized animals and immortalizing the
10 cells in conventional fashion, e.g. by fusion with myeloma
cells or by Epstein-Barr (EB)-virus transformation and
screening for clones expressing the desired antibody. The
hybridoma technique described originally by Koehler and
Milstein, Eur. J. Immunol., 6: 511 (1976) and also
15 described by Hammerling et al., In: Monoclonal Antibodies
and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981)
has been widely applied to produce hybrid cell lines that
secrete high levels of monoclonal antibodies against many
specific antigens.

20   The hybrid cell lines can be maintained in vitro in
cell culture media. The cell lines producing the
antibodies can be selected and/or maintained in a
composition comprising the continuous cell line in
hypoxanthine-aminopterin thymidine (HAT) medium. In fact,
25 once the hybridoma cell line is established, it can be
maintained on a variety of nutritionally adequate media.
Moreover, the hybrid cell lines can be stored and preserved
in any number of conventional ways, including freezing and
storage under liquid nitrogen. Frozen cell lines can be
30 revived and cultured indefinitely with resumed synthesis
and secretion of monoclonal antibody.

        The secreted antibody is recovered from tissue culture
supernatant by conventional methods such as precipitation,
ion- exchange chromatography, affinity chromatography, or
35 the like. The antibodies described herein are also
recovered from hybridoma cell cultures by conventional
methods for purification of IgG or IgM, as the case may be,
that heretofore have been used to purify these

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immunoglobulins from pooled plasma, e.g., ethanol or
polyethylene glycol precipitation procedures. The purified
antibodies are sterile filtered.

5   While routinely mouse monoclonal antibodies are used,
the invention is not so limited; in fact, human antibodies
can be used. Such antibodies can be obtained, for example,
by using human hybridomas (Cote et al., Monoclonal
Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985)).
In fact, according to the invention, techniques developed
10   for the production of chimeric antibodies (Cabilly et al.,
U.S. Patent No. 4,816,567, Morrison et al., Proc. Natl.
Acad. Sci., 81: 6851 (1984); Boulianne et al., Nature, 312:
643-646 (1984); Neuberger et al., Nature, 312: 604 (1984);
Neuberger et al., Nature, 314: 268-270 (1985); Takeda et
15   al., Nature, 314: 452 (1985); EP 184,187; EP 171,496; EP
173,494; PCT WO 86/01533; Shaw et al., J. Nat. Canc. Inst.,
80: 1553-1559 (1988); Morrison, Science, 229: 1202-1207
(1985); Oi et al., BioTechniques, 4: 214 (1986)) by
coupling an animal antigen-binding variable domain to a
20   human constant domain can be used; such antibodies are
within the scope of this invention. The term "chimeric"
antibody is used herein to describe a polypeptide
comprising at least the antigen binding portion of an ,
antibody molecule linked to at least part of another
25   protein (typically an immunoglobulin constant domain).

In one embodiment, such chimeric antibodies contain
about one third rodent (or other non-human species)
sequence and thus are capable of eliciting a significant
anti-globulin response in humans. For example, in the case
30   of the murine anti-CD3 antibody OKT3, much of the resulting
anti-globulin response is directed against the variable
region rather than the constant region (Jaffers et al.,
Transplantation, 41: 572-578 (1986)).

Humanized antibodies are used to reduce or eliminate
35   any anti-globulin immune response in humans. In practice,
humanized antibodies are typically human antibodies in
which some amino acid residues from the complementarity
determining regions (CDRs), the hypervariable regions in

the variable domains which are directly involved with
formation of the antigen-binding site, and possibly some
amino acids from the framework regions (FRs), the regions
of sequence that are somewhat conserved within the variable
5　domains, are substituted by residues from analogous sites
in rodent antibodies. The construction of humanized
antibodies is described in Riechmann et al., Nature, 332:
323-327 (1988), Queen et al., Proc. Natl. Acad. Sci. USA,
86: 10029-10033 (1989), Co et al., Proc. Natl. Acad. Sci.
10　USA, 88: 2869-2873 (1991), Gorman et al., Proc. Natl. Acad.
Sci., 88: 4181-4185 (1991), Daugherty et al., Nucleic Acids
Res., 19: 2471-2476 (1991), Brown et al., Proc. Natl. Acad.
Sci. USA, 88: 2663-2667 (1991), Junghans et al., Cancer
Res., 50: 1495-1502 (1990), Fendly et al., Cancer Res., 50:
15　1550-1558 (1990) and in PCT applications WO 89/06692 and WO
92/22653.

　　　　In some cases, substituting CDRs from rodent
antibodies for the human CDRs in human frameworks is
sufficient to transfer high antigen binding affinity (Jones
20　et al., Nature, 321: 522-525 (1986); Verhoeyen et al.,
Science, 239: 1534-1536 (1988)) whereas in other cases it
is necessary to additionally replace one (Riechmann et al.,
supra) or several (Queen et al., supra) FR residues. See
also Co et al., supra.

25　　　　The invention also encompasses the use of human
antibodies produced in transgenic animals. In this system,
DNA encoding the antibody of interest is isolated and
stably incorporated into the germ line of an animal host.
The antibody is produced by the animal and harvested from
30　the animal's blood or other body fluid. Alternatively, a
cell line that expresses the desired antibody can be
isolated from the animal host and used to produce the
antibody in vitro, and the antibody can be harvested from
the cell culture by standard methods.

35　　　　Particularly preferred for use in the methods of the
invention is the humanized anti-IgE antibody E25
(rhuMAbE25) as described in the Examples below. The
construction of rhuMAbE25 is disclosed in Presta et al., J.

Immunol., **151**: 2623-2632 (1993).

Anti-IgE antibody fragments can also be used in the methods of the invention. Any fragment of an anti-IgE antibody capable of blocking or disrupting IgE interaction
5   with immune cells or genetically engineered cells is suitable for use herein. Preferably, the antibody fragment is unable to trigger histamine release in immune cells or genetically engineered cells.

Suitable anti-IgE antibody fragments can be obtained
10 by screening combinatorial variable domain libraries for DNA capable of expressing the desired antibody fragments. These techniques for creating recombinant DNA versions of the antigen-binding regions of antibody molecules (known as F(ab) fragments), which bypass the generation of monoclonal
15 antibodies, are encompassed within the practice of this invention. One extracts antibody-specific messenger RNA molecules from immune system cells taken from an immunized animal, transcribes these into complementary DNA (cDNA), and clones the cDNA into a bacterial expression system.
20 One example of such a technique suitable for the practice of this invention was developed by researchers at Scripps/Stratagene, and incorporates a proprietary bacteriophage lambda vector system that contains a leader sequence that causes the expressed F(ab) protein to migrate
25 to the periplasmic space (between the bacterial cell membrane and the cell wall) or to be secreted. One can rapidly generate and screen great numbers of functional F(ab) fragments for those that bind the antigen. Such IgE-binding molecules (F(ab) fragments with specificity for the
30 IgE protein) are specifically encompassed within the term "antibody" as it is defined, discussed, and claimed herein.

In a further embodiment of the invention, soluble Fc$_\epsilon$RI receptor can be used as the IgE antagonist. Soluble Fc$_\epsilon$RI receptors suitable for use herein include molecules
35 comprising the IgE binding site in the extracellular domain (exodomain) of the Fc$_\epsilon$RI α chain. The α chain of Fc$_\epsilon$RI can be genetically modified such that the exodomain is secreted as a soluble protein in a recombinant expression system

according to the method of Blank et al., J. Biol. Chem., 266: 2639-2646 (1991) or Qu et al., J. Exp. Med., 167: 1195. The candidate soluble Fc$_\epsilon$RI receptor can be screened for activity as an IgE antagonist according to the above-

5   described methods for assaying the bioactivity of a agent in blocking IgE-induced immune cell sensitization. Alternatively, the candidate soluble Fc$_\epsilon$RI receptor can be screened for activity as an IgE antagonist by using the candidate soluble Fc$_\epsilon$RI receptor to block the interaction

10 between IgE and a genetically engineered cell in any of the methods for diagnosing allergy with genetically engineered cells described above.

         The invention also encompasses the use of IgE-binding peptides in addition to anti-IgE antibodies and soluble

15 Fc$_\epsilon$RI receptor. Any IgE-binding peptide capable of disrupting or blocking the interaction between IgE and Fc$_\epsilon$RI receptor is suitable for use herein. A candidate IgE-binding peptide can be screened for activity as an IgE antagonist according to the above-described methods for

20 assaying the bioactivity of an agent in blocking IgE-induced immune cell sensitization. Alternatively, the candidate IgE-binding peptide can be screened for activity as an IgE antagonist by using the candidate IgE-binding peptide to block the interaction between IgE and a

25 genetically engineered cell in any of the methods for diagnosing allergy with genetically engineered cells described above.

         Preferably, the IgE-binding peptide is unable to induce histamine release in immune cells or genetically

30 engineered cells. A candidate IgE-binding peptide can be tested by mixing sensitized immune cells (or sensitized genetically engineered cells) with the peptide instead of allergen in any of the above-described methods for diagnosing allergic disease. Sensitized cells mixed with

35 allergen or merely buffer serve as positive and negative controls, respectively. If the candidate IgE-binding peptide fails to induce histamine release that is substantially above the level of the negative control, the

peptide is acceptable for use in the methods of the
invention.

In addition to IgE antagonists which interfere with
IgE/Fc$_\epsilon$RI receptor interaction by binding to IgE, such as
5   anti-IgE antibodies, fragments thereof, soluble Fc$_\epsilon$RI
receptor and other IgE-binding peptides described above,
the invention encompasses the use of IgE antagonists which
disrupt IgE/Fc$_\epsilon$RI receptor interaction by competing with IgE
for binding to the Fc$_\epsilon$RI receptor, thereby lowering the
10   available Fc$_\epsilon$RI receptor.

IgE variant is one example of a Fc$_\epsilon$RI receptor-binding
competitor that is suitable for use in the methods of the
invention. IgE variants are forms of IgE possessing an
alteration, such as an amino acid substitution or
15   substitutions and/or an amino acid deletion or deletions,
which reduces or eliminates the ability of the altered IgE
molecule to sensitize immune cells and wherein the altered
IgE molecule is capable of competing with IgE for binding
to Fc$_\epsilon$RI receptor. A candidate IgE variant can be screened
20   for activity as an IgE antagonist according to the above-
described methods for assaying the bioactivity of an agent
in blocking IgE-induced immune cell sensitization.
Alternatively, the candidate IgE variant can be screened
for activity as an IgE antagonist by using the candidate
25   IgE variant to block the interaction between IgE and a
genetically engineered cell in any of the methods for
diagnosing allergy with genetically engineered cells
described above.

A candidate IgE variant can be tested for lack of
30   immune cell-sensitizing activity or genetically engineered
cell sensitizing activity by comparing the histamine
release of IgE variant-sensitized cells and the histamine
release of IgE-sensitized cells upon allergen challenge.
If the level of histamine release in the mixture containing
35   the candidate IgE variant is substantially lower than the
level of histamine release in the mixture containing IgE,
the variant is suitable for use in the methods of the
invention.

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Fragments of IgE variants are also suitable for use
herein. Any fragment of an IgE variant capable of
competing with IgE for binding to Fc,RI receptor can be used
in the methods of the invention. A candidate IgE variant
5   fragment can be tested for the desired activity according
to the methods for screening candidate IgE variants
described above.

The invention also encompasses the use of Fc,RI
receptor-binding peptides in addition to IgE variants and
10   fragments thereof. Any Fc,RI receptor-binding peptide
capable of disrupting or blocking the interaction between
IgE and Fc,RI receptor is suitable for use herein. A
candidate Fc,RI receptor-binding peptide can be screened for
activity as an IgE antagonist according to the above-
15   described methods for assaying the bioactivity of an agent
in blocking IgE-induced immune cell sensitization.
Alternatively, the candidate Fc,RI-binding peptide can be
screened for activity as an IgE antagonist by using the
candidate Fc,RI-binding peptide to block the interaction
20   between IgE and a genetically engineered cell in any of the
methods for diagnosing allergy with genetically engineered
cells described above.

Preferably, the Fc,RI receptor-binding peptide is
unable to induce histamine release in immune cells or
25   genetically engineered cells. A candidate Fc,RI receptor-
binding peptide can be tested for lack of immune cell
sensitizing activity (or genetically engineered cell
sensitizing activity) by the same methods described above
for testing the immune cell (or genetically engineered
30   cell) sensitizing activity of IgE variants.

Practice of the invention is not limited to the use of
peptide IgE antagonists. Any compound capable of
functioning as an IgE antagonist is suitable for use in the
methods of the invention, including non-proteinaceous small
35   molecule compounds. Such a compound can be tested for IgE
antagonist activity by use of the compound as the IgE
antagonist in the methods described above. If the compound
is unable to trigger the release of a pharmacological

mediator or mediators in sensitized immune cells (or
sensitized genetically engineered cells) and is capable of
disrupting or blocking the interaction between IgE and the
high affinity receptor Fc$_\epsilon$RI on immune cells (or genetically
5   engineered cells) such that the cells do not release a
pharmacological mediator or mediators in response to
allergen stimulus, the compound qualifies as an IgE
antagonist as defined, described and claimed herein.

     Further details of the invention can be found in the
10   following examples, which further define the scope of the
invention. All references cited herein are expressly
incorporated by reference in their entirety.

                      EXAMPLES
15

                     Example 1

1. MATERIALS AND METHODS

     a. Materials

     Monoclonal antibodies produced by conventional
20   hybridoma techniques were humanized and isolated as
described by Carter *et al.*, Proc Natl Acad Sci USA: 89:
4285-4289 (1992) and Presta *et al.*, J. Immunol, 151: 2623-
2632 (1993). These included murine anti-human IgE MAb,
(MAE1), which binds to IgE on basophils and triggers
25   histamine release (Presta *et al.*), humanized anti-human IgE
MAb (rhuMAbE25), humanized anti-HER2 MAb (rhuMAbHER2) and
humanized anti-CD18 MAb (rhuMAbCD18). Human IgG Fc
fragment was purchased from Organon Teknika Corp., West
Chester, PA. Ragweed antigens E-B & E-C (lot A-601-903A-
30   185) were obtained from the National Institute of Health,
Bethesda, MD. Dust mite allergen (*D. farinae* & *D.
pteronyssinus* 50/50 mix, lot 6691UM) was from Miles Inc.,
West Haven, CT. Ragweed specific human plasmas (RSHP) and
dust mite specific human plasma (DMSHP) were obtained from
35   North American Biological Company, Miami, FL. Hank's
balanced salt solution, (HBSS, 1X) was from Gibco BRL,
Gaithersburg, Md. Bovine serum albumin (BSA, Fraction V)
was obtained from Sigma, St. Louis, MO. CaCl$_2$·2H$_2$O and

MgCl$_2$·6H$_2$O were analytical grade reagents obtained from
Baker Chemical, Pittsburgh, NJ.

    b. <u>Blood Donors</u>

    Heparinized whole blood for the assays was obtained
5 from a group of non-allergic or allergic individuals. The
group consisted of seventeen women (non-pregnant) and
twenty-six men with age ranging from 22 to 51 and a mean
age of 34 ± 7. Volunteers were not on medication and
informed consent was obtained in all cases.

10   c. <u>Ragweed Allergen-Induced Histamine Release Assay</u>

    Whole blood (5 mL) from prescreened donors was drawn
into heparinized tubes (Becton Dickinson) and used in the
assay within 4 hours. Blood was diluted 1 : 7 with HBSS/1%
BSA. Nine parts of the diluted blood were added to one
15 part of RSHP and incubated with or without rhuMAbE25
standards (final concentrations ranging from 0.01 to 2
μg/mL) for 2 hours at 37°C in a humidified, 5% CO$_2$
incubator. After incubation, 100 μl aliquots of the
samples were transferred to a round bottom 96-well non-
20 tissue culture plate (Costar 3797) containing 50 μl buffer
(HBSS/1% BSA provided by the enzyme immunoassay kit),
ragweed allergen (0.3 μg/mL in PBS/0.01% BSA) or MAE1 (30
μg/mL in PBS/0.01% BSA) and further incubated for 30 min at
37°C. The incubation was stopped by transferring the plate
25 to ice. Plates were centrifuged at 900 x g for 5 min and
the supernatants harvested for histamine determination.
For each blood sample, total cellular histamine was
determined by mixing 50 μl of the sample with 950 μl
distilled water followed by two 15 minute cycles of
30 freezing and thawing. Samples were centrifuged at 900 x g
for 5 minutes and the supernatants were collected. The
concentration of histamine in the supernatant was
determined by a histamine enzyme immunoassay kit (AMAC,
Inc., Westbrook, ME) following the instructions of the
35 manufacturer. Briefly, histamine was acylated and allowed
to compete with histamine acetylcholinesterase for binding
to antibody coated onto microwells. After 18 hours, each
well was rinsed and the bound enzymatic activity was

measured by the addition of a chromogenic substrate (acetylthiocholine, dithionitrobenzoate). The intensity of the color developed at 405 nm was used to calculate the concentration of histamine in the sample with a standard

5   curve obtained with standards furnished by the kit. The calculated concentration of histamine was then converted to percent of total cellular histamine. Using histamine release by ragweed as the denominator, the percent inhibition of ragweed-induced histamine release by

10 rhuMAbE25 was calculated. The biological activity of various preparations of rhuMAbE25 was determined from the standard dilution curve in which percent inhibition of ragweed-induced histamine release was plotted against the concentration of rhuMAbE25 using a least square, non-linear

15 4 parameter fit program.

d. Divalent Cation Studies

The histamine release assay was performed in the same way as described in step c above except that the whole blood was diluted 1 : 7 in Hanks' balanced salt buffer

20 without Ca^{++} and Mg^{++} (HBSS^{--}) containing added $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, or $CaCl_2 \cdot 2H_2O$ plus $MgCl_2 \cdot 6H_2O$ at final concentrations of 0, 0.325, 0.625, 1.25, 2.5, 5 and 10 mM. After 2 hours of sensitization with 10% RSHP, the diluted blood was challenged with ragweed allergen (0.1 μg/mL) for

25 30 min at 37°C. The release of histamine in the supernatants was determined and converted to percent total cellular histamine.

e. Deuterium Oxide (D_2O) Studies

In screening blood donors for the assay, the effect of

30 deuterium oxide was determined. Heparinized whole blood from seventeen non-allergic donors were sensitized with 10% RSHP as in the assay format described above and then challenged with ragweed allergen prepared in either PBS/0.01%BSA or deuterium oxide (final concentration of

35 33.3%). The release of histamine in the supernatants after 30 min incubation was quantitated and converted to percent total cellular histamine.

f. Dust Mite Allergen-Induced Histamine Release Assay

The whole blood of a non-allergic donor diluted 1 : 7 with Hanks buffer was separately preincubated for 2 hr, either in the presence or absence of rhuMAbE25 (1 μg/mL), with seven plasmas (final concentration of 10%) taken from subjects allergic to dust mite and then challenged with the dust mite allergen according to the procedure described in step c above. The concentration of histamine in the supernatant was quantitated.

2. RESULTS

a. Inability of rhuMAbE25 to Trigger Histamine Release

A first study was performed to confirm that rhuMAbE25 does not bind to RSHP-sensitized basophils and trigger histamine release in the absence of antigen stimulation. Whole blood specimens from 12 donors were presensitized with 10% RSHP and challenged with 0.1 μg/mL of ragweed allergen, 10 μg/mL of MAE1 (a murine MAb which can bind to IgE on surface of basophils and trigger histamine release as described by Presta et al.), 10 μg/mL of rhuMAbE25, or HBSS/1% BSA. After incubation, the concentration of histamine in the supernatant was quantitated. Both ragweed allergen and MAE1 elicited histamine release. However, rhuMAbE25 did not induce histamine release above background levels (Fig.1).

b. Sensitization of Basophils with RSHP

In order to maximally sensitize basophils, seven human plasma samples taken from individuals known to be allergic to ragweed were screened for their ability to induce histamine release. RSHP lot#42-365054 (total IgE 1.64 μg/mL) consistently resulted in the highest histamine release after challenge with ragweed allergen (Fig. 2). Consequently, RSHP (lot 42-365054) was used to sensitize the basophils in all subsequent assays. As shown in Fig. 3, presensitization of basophils in whole blood with exogenous human plasma containing IgE specific for ragweed (RSHP) was a prerequisite for subsequent histamine release upon challenge with ragweed allergen. In the absence of

RSHP, only background levels of histamine were detected, while a dose-dependent release of histamine was observed in the presence of RSHP.

c. Donor Screens

5   Non-allergic volunteers were prescreened prior to being used as donors for the assay. The screening protocol involved presensitizing donors' whole blood with 10% RSHP in the absence and presence of rhuMAbE25 (10 μg/mL) for 2 hours at 37°C followed by challenge with ragweed allergen

10   (0.1 μg/mL). Those donors with basophils that released greater than 10% of the total cellular histamine were selected for further study. As shown in Fig. 4, the cells from some donors did not release histamine upon ragweed challenge and conversely the cells from other donors were

15   found to release histamine in the absence of allergen challenge. Of 43 volunteers, 12 were adequate 'responders' for routine use in this embodiment of the assay.

d. Kinetics of Histamine Release

The ragweed-induced histamine release from RSHP

20   sensitized whole blood was rapid and reached a plateau after 20-30 min incubation (Fig. 5). Based on these results, a thirty minute incubation at about 37°C was established as an optimal condition for the assay. The. release of histamine was significantly reduced if the

25   incubation was performed at ambient temperature.

e. Specificity

Preincubation of RSHP-sensitized whole blood with other humanized monoclonal antibodies, differing in complementary determining regions (CDR) but exhibiting an

30   overall homology of about 95% to rhuMAbE25, produced no inhibitory effect on histamine release elicited by ragweed allergen as shown in Table I below. In addition, a human IgG Fc fragment had no inhibitory effect on ragweed-induced histamine release.

35

Table I

Treatment	%Total Cellular Histamine in Supernatant	
	- Ragweed Challenge	+ Ragweed Challenge
Buffer	0.7%	53.6%
rhuMAbE25	0.6%	1.8%
rhuMAbHER2	0.5%	43.4%
rhuMAbCD18	0.5%	59.9%
huIgG Fc fragment	0.6%	63.9%

Concentration of antibody at 10 μg/mL
Concentration of rhuMAbE25 at 1 μg/mL

f. Variability

To determine interassay variability, a total of 23 assays were performed using blood from 12 donors. Results from these assays are tabulated in Table II below.

Table II

Experiment	%Inhibition of Ragweed-Induced Histamine Release		
	[rhuMAbE25] (μg/mL)		
	0.1	0.5	1
1	28.03	64.41	93.57
2	29.79	85.53	98.73
3	19.31	94.06	95.35
4	24.04	91.63	87.21
5	11.55	60.28	98.77
6	36.06	89.65	99.63
7	16.47	95.04	92.03
8	15.42	85.26	95.49
9	24.71	79.40	87.34
10	24.63	64.83	100.00
11	16.67	100.00	97.82
12	20.31	87.87	100.00
13	26.93	61.45	84.85
14	21.07	74.95	93.74
15	37.62	83.83	92.39
16	18.44	66.71	84.60
17	31.53	69.19	84.38
18	22.59	60.41	97.56
19	14.24	60.10	97.82
20	14.82	86.40	97.31
21	12.05	90.69	82.61
22	36.15	86.64	97.82
23	15.46	63.08	100.00

The percent inhibition of ragweed-induced histamine release by 0.1, 0.5 and 1 μg/mL rhuMAbE25 averaged 22.5% ± 7.8%, 78.3% ± 13.3% and 93.9 ± 5.8% respectively. The interassay variation (%CV) calculated from the percent

5 inhibition of histamine release by 0.5 μg/mL rhuMAbE25 was 17% (n = 23). Variability within a donor over a time period of 8 weeks was less, averaging 9.05% (n = 7).

g.   Divalent Cations

It has been well established that histamine release is

10 affected by Ca^{2+} in the incubation medium (Lichenstein and Osler, J. Exp. Med., 120: 507-530 (1964); Haydik and Ma, Clin. Rev. Allergy, 6: 141-162 (1988); MacGlashan and Botana, J. Immunol. 150: 980-991 (1993)). Consequently, the effects of Ca^{2+} and Mg^{2+} in the above-described assay

15 were determined. When whole blood was diluted in Hanks balanced salt buffer depleted of Ca^{2+} and Mg^{2+} ($HBSS^{--}$) there was a substantial decrease of releasibility of histamine compared to whole blood diluted in Hanks balanced salt buffer containing Ca^{2+} and Mg^{2+} (Fig. 6). Adding equal

20 concentrations of $CaCl_2 \cdot 2H_2O$ and $MgCl_2 \cdot 6H_2O$ to $HBSS^{--}$ restored histamine release.   While adding back $MgCl_2 \cdot 6H_2O$ alone partially restored release, the addition of $CaCl_2 \cdot 2H_2O$ completely restored the releasability of histamine (Fig. 7).

h.   Inhibition of Histamine Release by Preincubation

25   with Blocking Antibodies

Preincubation of ragweed specific serum with either 0.5 or 1 μg/ml rhuMAbE25 substantially inhibited the release of histamine subsequently stimulated by ragweed challenge (Fig. 5). The inhibitory effects of rhuMAbE25 are

30 best shown by plotting percent inhibition of ragweed-induced histamine release against the concentration of rhuMAbE25.   A typical rhuMAbE25 standard curve is shown in Fig. 8. Inhibition of ragweed-induced histamine release was dose-dependent and can often be attained by rhuMAbE25

35 above 1 μg/ml. The optimal assay range for rhuMAbE25 was from about 0.1 to about 1 μg/ml with mean IC_{50} of 0.255 ± 0.079 μg/ml (n = 15). The assay format can be readily modified to determine the effectiveness of rhuMAbE25 in

blocking histamine release elicited by other allergens. An example is shown in Fig. 9. The whole blood of a non-allergic donor was separately sensitized, either in the absence or presence of rhuMAbE25 (1 μg/ml), with 10% plasma

5 from subjects allergic to dust mite for 2 hr at 37°C. Next, the cells were challenged by the addition of dust mite allergen (100 ng/ml) for another 30 min. Histamine release in the supernatant was determined. It was observed that for patients B, C, F and G, the dust mite-induced histamine

10 release was inhibited by 1 μg/ml of rhuMAbE25. Patient A, D and E did not significantly elevate histamine release above background after the challenge with dust mite allergen. A higher concentration of rhuMAbE25 was required to completely block dust mite-induced histamine

15 release if 50%, as opposed to 10%, of the dust mite specific human plasma was used to sensitize the basophils. Thus, the assay format is useful in both demonstrating the presence of IgE specific for an allergen as well as the ability of an anti-IgE antibody to block the response

20 elicited by an allergen.

i. Deuterium Oxide Studies

It has been reported that deuterium oxide enhances histamine release from leukocytes (Gillespie and Lichtenstein, J. Clin. Invest. 51: 2941-2947 (1972)).

25 Therefore, the effect of deuterium oxide in the ragweed-induced histamine release was determined. D_2O alone did not trigger histamine release. As shown in Table III below, incorporation of D_2O in the incubation medium (final concentration of 33%) significantly enhances ragweed-

30 induced histamine release. Out of seventeen randomly selected non-allergic donors, 15 donors (88%) exhibited histamine release greater than 20% of total cellular histamine upon challenge with ragweed allergen in this assay format. There was a mean increase of histamine

35 release by 6.3 fold.

Table III

	%Total Cellular Histamine		
Donor No.	Ragweed	Ragweed in D_2O	No. of fold Increase
13	9.3%	37.8%	4.1
14	3.1%	30.3%	9.8
15	13.0%	25.1%	1.9
16	52.8%	75.4%	1.4
17	2.5%	29.9%	12.0
18	2.0%	26.7%	13.4
19	3.4%	18.0%	5.5
20	1.9%	39.5%	20.8
21	5.0%	25.6%	5.1
22	1.9%	14.4%	7.6
23	13.1%	39.8%	3.1
24	33.6%	35.7%	1.1
25	16.0%	43.0%	2.7
26	18.9%	77.7%	4.1
27	20.8%	53.5%	2.6
28	2.9%	28.9%	9.9
29	40.4%	72.2%	1.8
mean	14.2%	39.6%	6.3
N	17	17	17

Example 2

1.   MATERIALS AND METHODS

     a.   Materials

     Monoclonal antibodies produced by conventional
hybridoma techniques were humanized and isolated as
described by Carter et al., Proc. Natl. Acad. Sci., 89:
4285-4289 (1992) and Presta et al., J. Immunol., 5: 2623-
2632 (1993). These included humanized anti-human IgE MAb
(rhuMAbE25)(referred to as "variant 12" in Presta et al.),
humanized anti-HER2 MAb (rhuMAbHER2), humanized anti-CD18
MAb (rhuMAbCD18), and humanized anti-ICAM MAb (rhuMAbICAM).
Goat anti-human IgE(ε) HRP conjugate was purchased from
Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD.
Recombinant human nerve growth factor (rhuNGF), tumor
necrosis factor (rhuTNF), and interferon-gamma (rhuIFN-
gamma) were generated, cloned and sequenced at Genentech
(South San Francisco, CA). Ragweed antigens E-B & E-C (lot
A-601-903A-185) were obtained from the National Institute

of Health, Bethesda, MD. Standardized mite allergen (*D. farinae*) (cat. #6720UP, lot #E53L3533) and house dust mix allergen (cat. #4701ED, lot #C63J8308) were purchased from Miles Inc., Elkhart, IN. Standardized cat pelt allergen

5   (lot #3E00202) and *Alternaria tenuis* (lot #3J17242) allergen were obtained from Center Laboratories, Port Washington, NY. Human plasmas were obtained from North American Biological company, Miami, FL. Hank's Balanced Salt Solution (HBSS, 1X) and glutamine were purchased from

10 Gibco BRL, Gaithersburg, MD. Bovine serum albumin (BSA, Fraction V), o-phenylenediaminedihydrochloride (OPD) substrate and Triton X-100 were purchased from Sigma, St. Louis, MO. Fetal bovine serum was purchased from Hyclone, Logan, Utah. Deuterium oxide (D_2O, 99.996% purity) was

15 obtained from Aldrich Chem. Company, Milwaukee, WI.

    b.   Blood donors

    Heparinized whole blood was collected from a group of healthy, non-allergic or allergic individuals who were prescreened prior to being used as donors for the human

20 basophil histamine assay (HBHA). The screening protocol involved presensitizing donors' whole blood with 10% RSHP in the absence or presence of rhuMAbE25 (10 μg/mL) for 2 hours at 37°C followed by challenge with ragweed allergen (0.1 μg/mL). Those donors with basophils that released

25 greater than 10% of the total cellular histamine were selected for further study. No volunteers were on medication and informed consent was obtained in all cases.

    c.   Human Basophil Histamine Assay (HBHA)

    Heparinized whole blood was diluted 1/7 with HBSS/1%

30 BSA and mixed with 10% (v/v) allergen sensitized human plasma (e.g. ragweed specific human plasmas (RSHP). The mixture was incubated with or without rhuMAbE25 (prepared in PBS/0.01% BSA) for 2 hours at 37°C in a humidified 5 % CO_2 incubator. After incubation, samples were transferred

35 to a 96-well plate (Costar #3797) containing either buffer (HBSS/1% BSA or 50% D_2O/0.8% NaCl/1.3 mM $CaCl_2$) or allergen (0.3 μg/mL in PBS/0.01% BSA or 50% D_2O/0.8% NaCl/1.3 mM $CaCl_2$) and further incubated for 30 minutes at 37°C. Plates

were centrifuged at 900 X g for 5 min at 4°C and the
supernatants were harvested for histamine determination.
For each blood sample, total cellular histamine was
determined by mixing 50 μL whole blood with 950 μL

5   distilled water followed by two 15 min cycles of freezing
and thawing. The concentration of histamine in the
supernatant was determined with a histamine enzyme
immunoassay kit (AMAC, Westbrook, ME).

d.   Cell culture

10 The RBL48 rat mast cell line was obtained as described
by Gilfillan et al., supra. The RBL48 cell line was
derived by transfecting parental rat mast cell line RBL2H3
with the human α-subunit of the high affinity IgE receptor
Fc$_r$RI (Gilfillan et al.). Cells were grown in sIMDM

15 (Iscove's modified Dulbecco's media supplemented with 10%
fetal bovine serum, 2 mM glutamine, and 500 μg/mL of active
geneticin (Gibco BRL #11811-031)) in a T175 tissue culture
flask (Falcon #3028) at 37°C in a humidified 5 % CO_2
incubator. The cells were harvested by exposure to 4 mL of

20 PBS/0.05 % trypsin/0.53 mM EDTA (Gibco BRL #15400-013) for
2 min at 37°C followed by centrifugation (400 X g for 10
min) and resuspension in fresh sIMDM. The cells were
optimally split at 1:5 ratio after confluence was attained.

e.   Human plasma IgE binding ELISA assay

25 RBL48 cells, seeded at 40,000 cells per well, were
cultured overnight at 37°C/5 % CO_2 in a humidified
incubator. After three washes with PBS/0.05 % Tween 20 in
a plate washer, the cells were fixed for 2 min with 200 μL
per well of absolute alcohol at ambient temperature

30 followed by six washes to remove residual alcohol. sIMDM
containing 10% RSHP with or without rhuMAbE25 (10 μg/mL,
final concentration) were incubated with cells for 1 hr at
37°C. After the incubation, cells were rinsed 6 times with
PBS/0.05 % Tween 20. The captured IgE were then reacted

35 for 30 min at 37°C with goat anti-human IgE-HRP followed by
30 min incubation with OPD substrate at ambient
temperature. Reaction was stopped by the addition of 100
μL/well of 6 N H_2SO_4. Absorbance at 492 nm was measured.

f.   Rat Mast Cell Histamine Assay (RMCHA)

RBL48 cells were grown to confluence and then trypsinized as described in section d above. Cells in suspension were counted with a hemacytometer (Reichert-Jung

5 Company, Buffalo, NY) and the density was adjusted to 0.4 X 10^6 cells/mL. Cells were then seeded at 100 μL/well (40,000 cells per well) in a 96-well, U-shaped plate (Linbro #76-042-05) and cultured for 24 hr at 37°C in a humidified 5 % CO_2 incubator. After being washed once with 200 μL/well of

10 sIMDM, the cells were preincubated for 2 hours with 100 μL/well of fresh sIMDM including 3 U/mL of sodium heparin (Becton Dickinson #367673) and 10% (v/v) of allergen sensitized human plasma containing allergen specific IgE either in the presence or absence of rhuMAbE25 standards

15 (final concentrations ranging from 0.078 to 10 μg/mL) at 37°C. After incubation, culture medium in the well was removed by aspiration and adherent cells were washed 3 times with sIMDM. The cells were then incubated with 100 μL/well of histamine release buffer (HRB) (50% $D_2O/0.8$%

20 NaCl/1.3 mM $CaCl_2$/sIMDM), allergen (0.1 μg/mL in HRB), or 0.5 % triton solution (for total histamine release) for 30 min at 37°C. Plates were centrifuged at 900 X g for 5 min at 4°C and the supernatants were harvested and diluted 1000 times in PBS for total histamine release and 80 times for

25 allergen-induced histamine release. The concentration of histamine in the supernatant was determined as described for the HBHA assay in section c above.

g.   Correlation study of RMCHA and HBHA

15 samples of rhuMAbE25 (stored at -70°C, 2-8°C, 25 °C,

30 and 40 °C for 1 to 18 months)were tested in parallel for the ability to inhibit ragweed-induced histamine release in both the HBHA and the RMCHA. Two concentrations (0.625 and 1.25 μg/mL for RMCHA, 0.25 and 0.5 μg/mL for HBHA) of each sample were used in the assays as described in sections c

35 and f above. Protein concentrations of samples were determined from the rhuMAbE25 standard curve. The recovered concentrations (after correction for the dilution factor) of samples were calculated.

In a separate study, a panel of 5 allergens (prepared in 50% glycerin) that included mite (*D. farinae*), house dust mix, ragweed, cat pelt, and *Alternaria tenuis* was used to screen a panel of 8 human plasmas (7 allergic individual

5 plasma samples from North American Biological company, Miami, FL and 1 pooled plasma sample from Genentech Inc., South S.F., CA) in both the RMCHA and the HBHA. The RBL48 rat mast cells and the naive donor blood basophils were first sensitized for 2 hr at 37°C with 10% (v/v) human

10 plasma in the presence or absence of rhuMAbE25 (1 μg/mL, prepared in sIMDM/3 U/mL sodium heparin for RMCHA and in PBS/0.01% BSA for HBHA), then challenged with buffer (for HBHA, 33% D_2O/0.8% NaCl/1.3 mM $CaCl_2$, for RMCHA, 33% D_2O/0.8% NaCl/1.3 mM $CaCl_2$/sIMDM) or a single dose of

15 allergen (at final concentration of 0.1 μg/mL, assuming 333.3 AU/mL=33.33 BAU/mL=100 PNU/mL=1 μg/mL) in buffer with 33% D_2O. Histamine concentration in the supernatant was determined as described above.

h.   Data Analysis

20   Histamine concentration was determined from the histamine standard curve using the "Read" computer program. Percent inhibition was calculated by MicroSoft Excel 4.0 program. Graphs were drawn using the KaleidaGraph 3.0 · program. Statistics were analyzed in StatView 4.0 program.

25 2.   Results

a.   Binding of IgE to RBL48 cells

The binding of IgE to RBL48 cells was evaluated in an IgE binding ELISA which uses an alcohol-fixed RBL48 monolayer to determine whether human IgE in the RSHP (total

30 IgE measured at 1.64 μg/mL) could bind to the α-subunit of the $Fc_{\epsilon}RI$ receptors expressed on the surface of the RBL48 cells. Fig 10 demonstrates that the IgE present in the RSHP does indeed bind to the $Fc_{\epsilon}RI$ receptors of the RBL48 cells. In addition, this binding is specific to IgE since

35 the addition of a blocking anti-IgE monoclonal antibody, rhuMAbE25, completely abolishes the binding.

b   Specificity of the RMCHA

A RMCHA was performed (Table IV) to demonstrate that
rhuMAbE25, like other recombinant human monoclonal
antibodies such as anti-HER2, anti-CD18, and anti-ICAM and
5   recombinant human cytokines such as IFN-γ, TNF, and NGF,
when applied alone to the RBL48 cells, failed to induce
mast cell degranulation. RBL48 cells were seeded at 40,000
cells/well and were cultured overnight at 37°C in a 5% CO_2
incubator.   Cells were preincubated for 2 hours with either
10 sIMDM/50% D_2O, rhuMAbE25 (10 μg/mL), rhuMAbHER2 (10 μg/mL),
rhuMAbCD18 (10 μg/mL), rhuMAbICAM (10 μg/mL), rhuIFN-γ (10
μg/mL), rhuTNF (10 μg/mL), or rhuNGF (10 μg/mL) in the
presence of 10% (v/v) RSHP at 37°C. After three washes with
sIMDM, cells were challenged with 0.1 μg/mL of ragweed
15 allergen in the presence of 50% D_2O for 30 min at 37°C. In
addition, RSHP-sensitized cells were directly challenged
with either rhuMAbE25 (10 μg/mL), rhuMAbHER2 (10 μg/mL),
rhuMAbCD18 (10 μg/mL), rhuMAbICAM (10 μg/mL), rhuIFN-γ (10
μg/mL), rhuTNF (10 μg/mL), or rhuNGF (10 μg/mL) alone for
20 30 min at 37°C. Each of the values shown in Table IV below
represents the mean of triplicate determinations.

Table IV.

25 Specificity of RMCHA for rhuMAbE25

Treatment supernatant	% Total cellular histamine in	
	− Ragweed challenge	+Ragweed challenge
Buffer	2.0 ± 0.2	22.0 ± 0.1
rhuMAbE25	2.7 ± 0.5	2.1 ± 0
rhuMAbHER2	2.0 ± 0.3	23.6 ± 0.1
rhuMAbCD18	2.3 ± 1.2	22.9 ± 0.7
rhuMAbICAM	1.7 ± 0	23.7 ± 1.4
rhuIFN-γ	2.3 ± 0.9	23.3 ± 1.1
rhuTNF	1.6 ± 0.1	23.9 ± 0.2
rhuNGF	2.0 ± 0.5	22.9 ± 0.7

No inhibition of histamine release was observed when
samples were preincubated with 10 μg/mL of the

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aforementioned antibodies and cytokines. Moreover,
preincubation of ragweed specific human plasma with 10
μg/mL of rhuMAbE25 completely abolished histamine release
induced by 0.1 μg/mL of ragweed in the presence of 50% D_2O.

5   c.   Effect of temperature and calcium

          Histamine release induced by ragweed in the RMCHA was
dependent on the concentration of ragweed, temperature, and
calcium ions (Fig. 11). When the ragweed challenge step
was incubated in the presence or absence of 50% D_2O at 37°C,
10   a bell-shaped dose-dependent histamine release curve that
reaches maximal release at 0.1 μg/mL was observed. The
release of histamine elicited by ragweed was attenuated or
abolished if the incubation was performed either in the
presence of 2.5 mM EDTA or at ambient temperature.

15   d.   Time course of histamine release

          The data presented in Fig. 12 demonstrates that
ragweed-induced histamine release at 37°C is time-dependent
and reaches maximal release at 30 min incubation. When the
incubation time was increased to 45 min or 60 min, the
20   concentration of histamine in the supernatant dropped
sharply from 36% to 16% of total cellular histamine. In
addition, preincubation of ragweed specific plasma with
either 0.5 or 1 μg/mL of rhuMAbE25 inhibited the release of
histamine induced by ragweed allergen. A typical standard
25   curve for quantitating the biological activity of rhuMAbE25
is shown in Fig. 13. Inhibition of ragweed-induced
histamine release by rhuMAbE25 has a mean IC_{50} of 1.19 ±
0.31 μg/mL (n=25).

          e.   Correlation of RMCHA with HBHA

30   In evaluating the activity of 15 samples of rhuMAbE25,
a good correlation was demonstrated between the RMCHA and
the HBHA with a correlation coefficient of 0.93 (n=59,
p<0.0001) (Fig. 14).

          In addition, a panel of five allergens was used to
35   challenge both naive donor blood basophils and RBL48 mast
cells presensitized with IgE from each of the 8 human
plasmas in the presence or absence of rhuMAbE25. A
representative bar graph of four individual plasma samples

for the RMCHA is shown in Fig. 15. When cells were
sensitized with plasma sample one (P1),only mite (D.
farinae) and ragweed allergen elicited histamine release
significantly above the baseline (33% D_2O/0.8% NaCl/1.3 mM
5 $CaCl_2$/sIMDM (HRB)). However, preincubation with a second
plasma sample (P2) caused significant histamine release
when induced by mite (D. farinae), house dust, ragweed, cat
pelt, and Alternaria tenuis allergens. Likewise,
preincubation with a third plasma sample (P3) caused
10 significant histamine release when induced by mite (D.
farinae), house dust, ragweed, and cat pelt. In contrast
none of the five allergens stimulated significant histamine
release when cells were preincubated with a fourth plasma
sample (P4). In all cases, this allergen-induced histamine
15 release could be blocked by 1 µg/mL of rhuMAbE25.
Histamine release data obtained from the blood and cell
assays performed on all eight plasma samples were analyzed
with simple regression statistics. Table V below shows the
correlation of the two assays for house dust allergen
20 (r=0.84, p= 0.0013, N=11), ragweed allergen (r=0.86, p=
0.0133, N=7), cat pelt allergen (r=0.67, p= 0.0165, N=12),
and all allergens (r=0.69, p= 0.0001, N=37).

Table V.

25

Correlation between HBHA and RMCHA in the allergen-induced
histamine release

Allergens	correlation coefficient (r)	N	p	slope
House dust	0.84	11	0.0013	3.52
Ragweed	0.86	7	0.0133	1.07
Cat pelt	0.67	12	0.0165	3.93
All allergens	0.69	37	0.0001	2.03

f.   Effect of deuterium oxide (D_2O)

40   In order to study the effect of 33% D_2O, if any, in the
HBHA system, a comparative study of ragweed-induced
histamine release in the presence or absence of 33% D_2O was
performed. A total of 27 randomly selected healthy donors

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were recruited for the study. Donors' heparinized whole
blood was pre-sensitized with 10% RSHP for 2 hr at 37°C
prior to challenge with 0.1 μg/mL (final concentration) of
ragweed allergen for a further 30 min in the presence or
5 absence of 33% D_2O. The concentration of histamine released
into the supernatant was converted to percent total
cellular histamine. Regression analysis shows a linear
correlation (r=0.80, slope=1.04, p= 0.0001) between
ragweed- and ragweed/33% D_2O- induced histamine release
10 (Fig. 16). The data indicate that the profile of enhanced
histamine release in the presence of 33% D_2O is proportional
and comparable to ragweed challenge without D_2O.

The effect of D_2O on the RMCHA was also examined. It
was determined that D_2O enhanced histamine release in RBL48
15 cells in a dose-dependent fashion reaching a maximal
release at 50% D_2O (Fig. 17). However, D_2O neither
stimulates histamine release by itself nor interferes with
rhuMAbE25 inhibition of histamine release by RBL48 cells.

The effect of D_2O on the pH of reagents used in these
20 assays was investigated in order to determine whether the
enhanced histamine release caused by D_2O is not due to
changes of pH. The pH of various reagents (2 mL) used in
the HBHA (HBSS, HBSS+1% BSA, HBSS+1% BSA+Blood+10% RSHP,
HBSS+1% BSA+Blood+10% RSHP+33% D_2O, Blood, and 100% RSHP)
25 and in the RMCHA (sIMDM, sIMDM+3 U/mL Sodium Heparin, and
sIMDM + 33% D_2O) were analyzed after 1 hr incubation at
either ambient or 37°C. Each pH value shown in Table VI
below represents the mean of triplicate determinations. As
expected, the pH of the reagents becomes slightly acidic at
30 37°C compared to that at ambient temperature. Although 33%
D_2O causes a slight alkalization of blood reagent (at 37°C)
from pH of 7.20 ± 0.01 to 7.34 ± 0.01, it does not
significantly change the pH of whole blood (pH 7.35 ± 0.01)
or the pH of the RMCHA reagents.

35

Table VI.

pH of various reagents for HBHA and RMCHA

Reagents	pH	
	Ambient	37°C
HBSS	7.20 ± 0.01	6.83 ± 0.01
HBSS+1% BSA	7.18 ± 0.01	6.82 ± 0.01
HBSS+1% BSA+Blood+RSHP	7.61 ± 0	7.20 ± 0.01
HBSS+1% BSA+Blood+RSHP+D_2O	7.75 ± 0	7.34 ± 0.01
Blood	7.51 ± 0.01	7.35 ± 0.01
RSHP	8.14 ± 0	7.95 ± 0.01
sIMDM	7.72 ± 0.03	7.44 ± 0.02
sIMDM+Sodium Heparin	7.73 ± 0.02	7.49 ± 0.01
sIMDM+D_2O	7.69 ± 0.01	7.42 ± 0.02

3.   Discussion

A bioassay was developed to quantitate allergen-
induced histamine release and rhuMAbE25 activity using a
rat mast cell line (RBL48) transfected with the α-subunit
of the human $Fc_{\epsilon}RI$ receptor. In the assay, RBL48 cells were
sensitized with human plasma containing allergen specific
IgE which binds to $Fc_{\epsilon}RI$ receptors expressed on the surface
of these cells (Fig. 10). Subsequent challenge with
ragweed allergen resulted in histamine release (Fig. 11)
that was inhibited by the anti-IgE monoclonal antibody
(rhuMAbE25) in a dose-dependent manner (Fig. 13). Since
rhuMAbE25 can inhibit any allergen-induced histamine
release, the format of this assay can be applied to all
allergens. The assay can be modified by using a panel of
allergens to screen human plasma samples for diagnosing
IgE-mediated allergic conditions (Fig. 15).

In developing the RMCHA, the effect of D_2O on histamine
release was carefully investigated. As shown in Fig. 17,
histamine release was not observed even when RBL48 cells
were challenged with high concentrations (up to 100%) of
D_2O. The data in Fig. 16 demonstrates a statistical
significant correlation ($r=0.80$, $n=27$, $p= 0.0001$) between
histamine release induced by ragweed and ragweed/33% D_2O
indicating that D_2O facilitates proportional histamine

release in blood basophils. The presence of 33% D_2O appears
to have no marked effect on the physiological pH of either
the HBHA or the RMCHA reagent (Table VI).

The format of RMCHA may be readily modified to
5   determine the effectiveness of rhuMAbE25 or other IgE
antagonists in inhibiting histamine release induced by a
variety of allergens as shown in Fig. 15. Histamine
release above baseline (HRB) is an indication of the
presence of allergen specific IgE in the plasma. The data
10 obtained herein indicates that plasma sample one (P1)
contains IgE specific to mite (*D. farinae*) and ragweed
since these two allergens stimulated histamine release
significantly above baseline. Likewise, plasma sample two
(P2) contains IgE specific to mite (*D. farinae*), house dust
15 mix, ragweed, cat pelt, and *Alternaria tenuis*; plasma
sample three (P3) contains IgE specific to mite (*D.
farinae*), house dust mix, ragweed, and cat pelt; and plasma
sample four (P4) contains no IgE specific to any of these
five allergens. Similar profiles of histamine release were
20 obtained when these allergens were used to challenge blood
basophils in the HBHA. Since the RMCHA correlates well
with the HBHA in evaluating the biological activities of
fifteen humanized anti-IgE monoclonal antibodies (Fig. 14)
and in histamine release induced by various allergens
25 (Table VI), it can be used as an additional tool of
diagnosing IgE-mediated allergies.

Since several studies have clearly demonstrated that
IgE levels correlate with allergic disease (Bahna, <u>Ann.
Allergy</u> <u>62</u>:471-475 (1989); Mascia *et al.*, <u>Ann. Allergy</u>
30 <u>62</u>:311-318(1989); Platts-Mills, <u>Am. Rev. Respir. Dis.</u> <u>145</u>:
S44-S47(1992), this RMCHA can be used to study and explore
IgE-mediated pathway as well as to predict the likely
efficacy of immunotherapy aimed at blocking of binding of
IgE to $Fc_{\epsilon}RI$.

35

CLAIMS

1.   A method for diagnosis of allergic disease in a patient comprising

5   (a) comparing release of a pharmacological mediator in a releasing mixture with release of the pharmacological mediator in a blocked releasing mixture, wherein the releasing mixture and the blocked releasing mixture each comprises a portion of a single tissue sample
10   from a naive donor, and wherein the releasing mixture and the blocked releasing mixture each further comprises a portion of a single serum sample from the patient, the presence or absence in the serum sample of IgE specific for an allergen of interest being theretofore unknown, and
15   wherein the blocked releasing mixture further comprises an IgE antagonist, and wherein both the releasing mixture and the blocked releasing mixture are admixed with the allergen; and

           (b) determining, based on the comparison in step
20   (a), the presence or absence in the serum sample of IgE specific for the allergen.

2.   The method of claim 1 wherein the pharmacological mediator is histamine.
25

3.   The method of claim 1 wherein the tissue sample is a blood sample.

4.   The method of claim 1 wherein the IgE antagonist
30   is an anti-IgE antibody or fragment thereof.

5.   The method of claim 4 wherein the anti-IgE antibody is a monoclonal anti-IgE antibody.

35   6.   The method of claim 5 wherein the monoclonal anti-IgE antibody is rhuMAbE25.

7.   The method of claim 1 wherein the IgE antagonist is an IgE variant or fragment thereof.

8.   The method of claim 1 wherein the IgE antagonist is a soluble Fc$_\epsilon$RI receptor.

9.   The method of claim 1 wherein the IgE antagonist is an IgE-binding peptide or a Fc$_\epsilon$RI receptor-binding peptide.

10.   The method of claim 1 wherein the tissue sample in the blocked releasing mixture is first admixed with the IgE antagonist and then admixed with the serum sample from the patient.

11.   The method of claim 1 wherein the serum sample in the blocked releasing mixture is first admixed with the IgE antagonist and then admixed with the tissue sample.

12.   A method for assaying the bioactivity of an agent for blocking IgE-induced sensitization of basophils, comprising
(a) comparing release of a pharmacological mediator in a releasing mixture with release of the pharmacological mediator in a blocked releasing mixture, wherein the releasing mixture and the blocked releasing mixture each comprises a portion of a single sample of whole blood obtained from a naive donor, and wherein the releasing mixture and the blocked releasing mixture each further comprises a portion of a single serum sample containing IgE specific for an allergen, and wherein the blocked releasing mixture further comprises the agent, and wherein both the releasing mixture and the blocked releasing mixture are admixed with the allergen; and
(b) determining, based on the comparison in step (a), the bioactivity of the agent for blocking IgE-induced sensitization of basophils.

13.   The method of claim 12 wherein the agent is a candidate anti-allergy therapeutic.

5   14.   The method of claim 13 wherein the candidate anti-allergy therapeutic is selected from the group consisting of anti-IgE antibodies or fragments thereof, IgE variants or fragments thereof, soluble $Fc_\epsilon RI$ receptors, IgE-binding peptides and $Fc_\epsilon RI$ receptor-binding peptides.

10

15.   The method of claim 12 wherein the agent is a candidate IgE antagonist.

16.   The method of claim 15 wherein the candidate IgE
15   antagonist is selected from the group consisting of anti-IgE antibodies or fragments thereof, IgE variants or fragments thereof, soluble $Fc_\epsilon RI$ receptors, IgE-binding peptides and $Fc_\epsilon RI$ receptor-binding peptides.

20   17.   The method of claim 12 wherein the whole blood sample in the blocked releasing mixture is first admixed with the agent and then admixed with the serum sample.

18.   The method of claim 12 wherein the serum sample
25   in the blocked releasing mixture is first admixed with the agent and then admixed with the whole blood sample.

19.   A method for diagnosis of allergic disease in a patient comprising
30   (a) comparing release of a pharmacological mediator in a reaction mixture with release of the pharmacological mediator in a blocked reaction mixture, wherein
         (1) the reaction mixture and the blocked reaction mixture comprise genetically engineered cells that are the
35   progeny of a common parent cell selected from the group consisting of: any mast cell host genetically engineered to display surface expression of a $Fc_\epsilon RI$ α-subunit that is capable of mediating the host cell's release of the

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pharmacological mediator upon induction by patient serum
IgE and allergen, and any basophil cell host genetically
engineered to display surface expression of a $Fc_\epsilon RI$ α-
subunit that is capable of mediating the host cell's
5   release of the pharmacological mediator upon induction by
patient serum IgE and allergen,

        (2) the reaction mixture and the blocked reaction
mixture each further comprises a portion of a single serum
sample from the patient, the presence or absence in the
10   serum sample of IgE specific for an allergen of interest
being theretofore unknown,

        (3) the blocked reaction mixture further comprises an
IgE antagonist, and

        (4) both the reaction mixture and the blocked reaction
15   mixture are admixed with the allergen; and

        (b) determining, based on the comparison in step (a),
the presence or absence in the serum sample of IgE specific
for the allergen.

20   20.   The method of claim 19 wherein the genetically
engineered cells are genetically engineered mast cells.

21.   The method of claim 20 wherein the genetically
engineered mast cells are genetically engineered rodent
25   mast cells.

22.   The method of claim 21 wherein the genetically
engineered rodent mast cells are genetically engineered rat
mast cells.

30

23.   The method of claim 22 wherein the genetically
engineered rat mast cells are RBL48 cells.

24.   The method of claim 19 wherein the $Fc_\epsilon RI$ α-subunit is
35   derived from the animal species of the patient.

25.   The method of claim 19 wherein the patient is human.

26.   The method of claim 19 wherein the Fc.RI α-subunit is derived from a human source.

27.   The method of claim 19 wherein the Fc.RI α-subunit is
5 heterologous to the genetically engineered cells.

28.   The method of claim 19 wherein the IgE antagonist is an anti-IgE antibody or fragment thereof.

10 29.   The method of claim 28 wherein the anti-IgE antibody is a monoclonal anti-IgE antibody.

30.   The method of claim 29 wherein the anti-IgE antibody is rhuMAbE25.

15

31.   The method of claim 19 wherein the IgE antagonist is an IgE variant or fragment thereof.

32.   The method of claim 19 wherein the IgE antagonist is a
20 soluble Fc.RI receptor.

33.   The method of claim 19 wherein the IgE antagonist is an IgE-binding peptide or a Fc.RI receptor-binding peptide.

25 34.   The method of claim 19 wherein the genetically engineered cells in the blocked reaction mixture are first admixed with the IgE antagonist and then admixed with the serum sample from the patient.

30 35.   The method of claim 19 wherein the serum sample in the blocked reaction mixture is first admixed with the IgE antagonist and then admixed with the genetically engineered cells.

35 36.   The method of claim 19 wherein the pharmacological mediator is histamine.

FIG. I

FIG. 2

RAGWEED-SENSITIZED HUMAN PLASMA

FIG. 3

FIG. 4

FIG. 5

FIG. 6

FIG. 7

FIG. 8

$IC_{50} = 0.221$ µg/ml

SLOPE = 1.93

% INHIBITION OF HISTAMINE RELEASE

[rhuMAb-E25] (µg/ml)

FIG. 9

HISTAMINE RELEASE (nM)

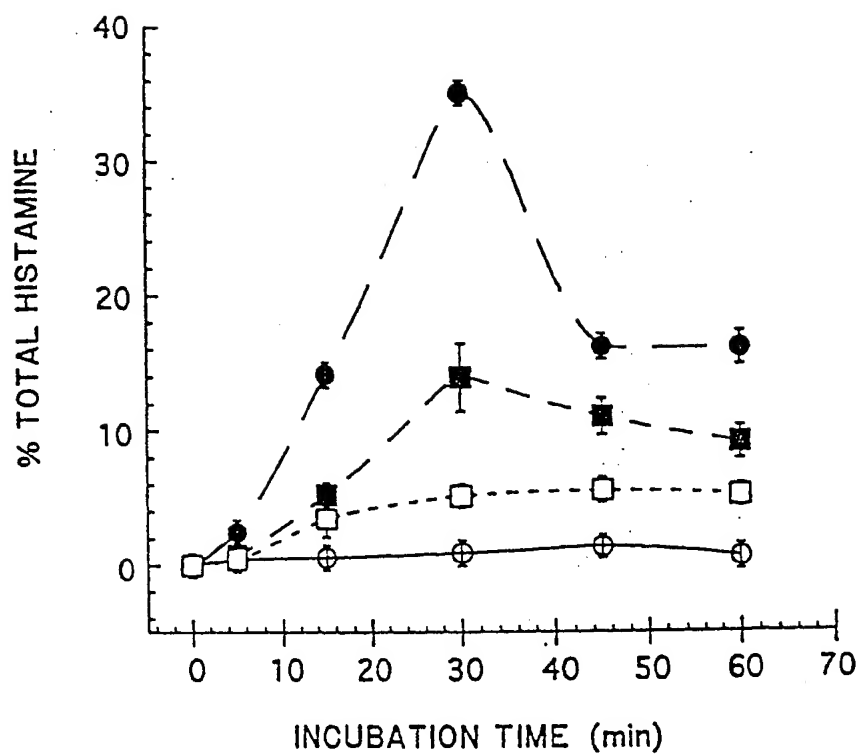HRB   A   B   C   D   E   F   G

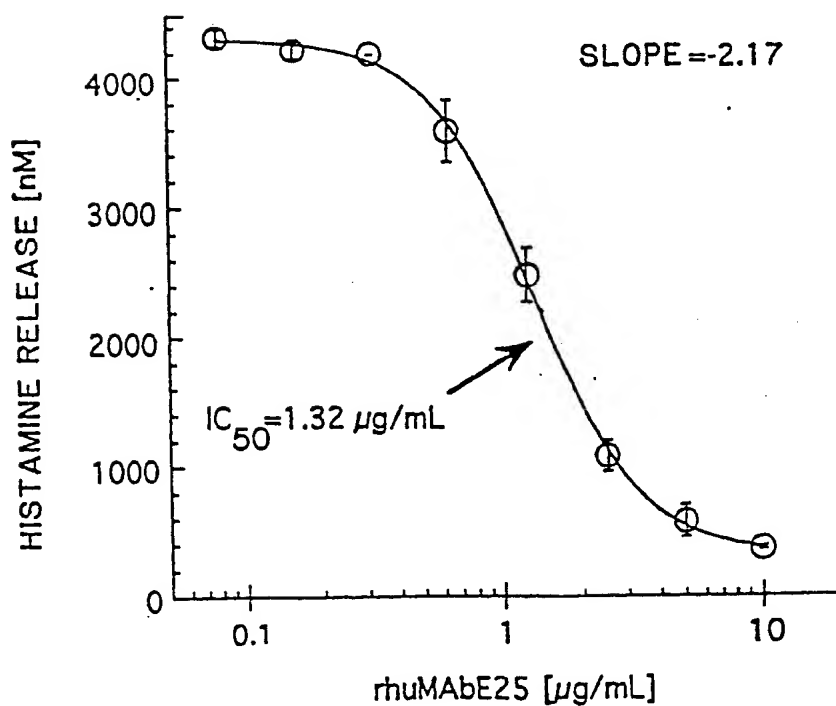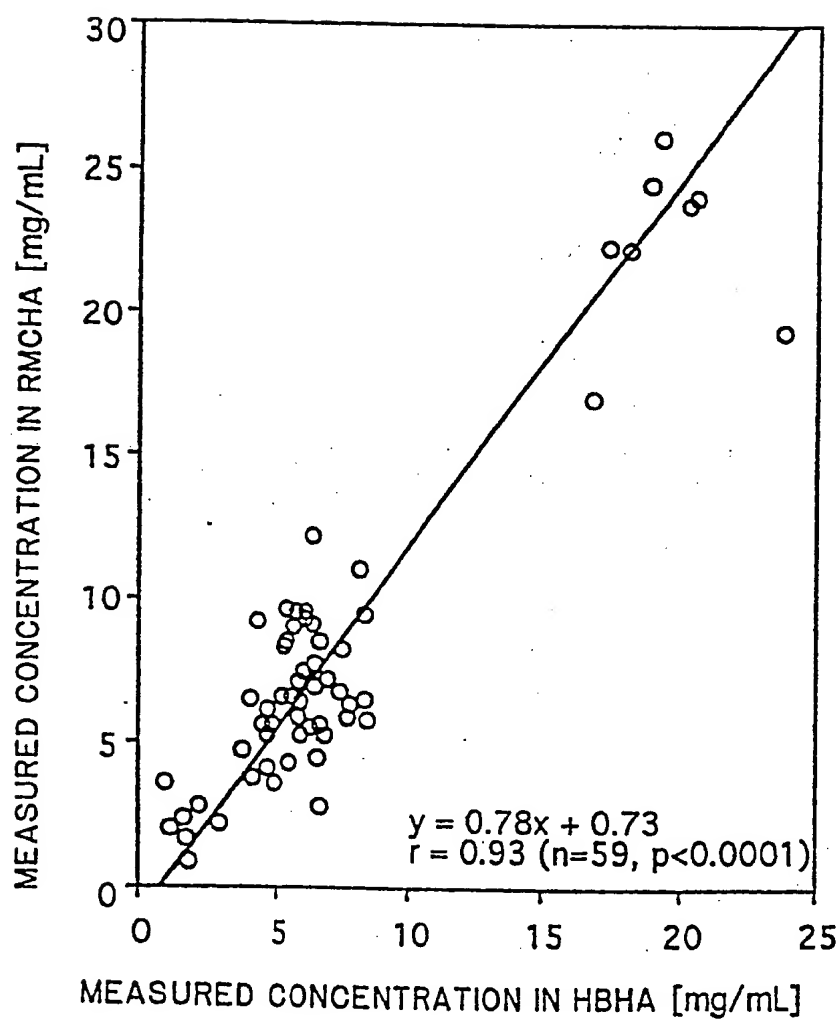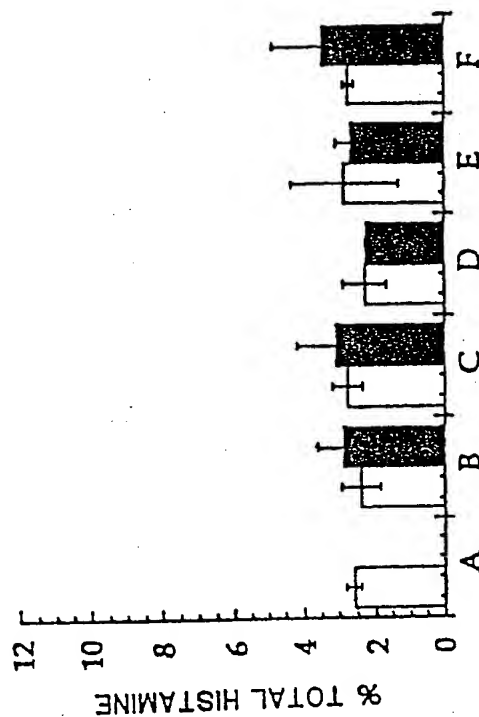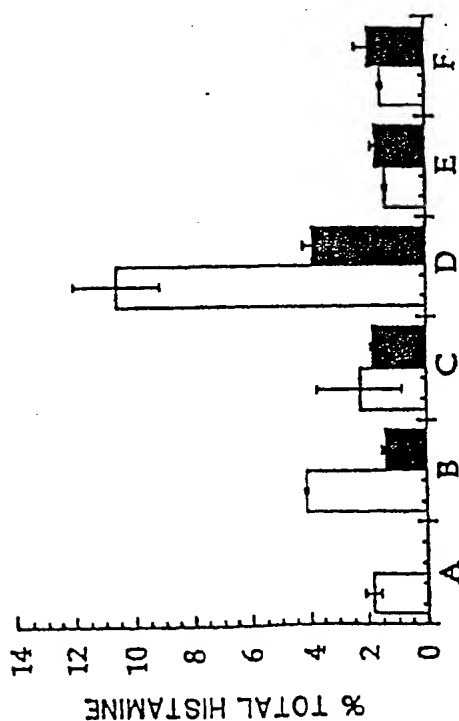DUST MITE-SENSITIZED HUMAN PLASMA

FIG. 10

FIG. 11

FIG. 12

FIG. 13

7 / 1 1

FIG. 14

FIG. 15A

FIG. 15B

FIG. 15C

FIG. 15D

FIG. 16

FIG. 17